

**Studies on the Role of Jaagsiekte Retrovirus in the
Aetiology of a Contagious Lung Tumour of Sheep**

Thesis submitted for the Degree of Doctor of Philosophy

by

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Declaration

This thesis has been composed by myself and describes my own work. The subject of the thesis was part of a larger project on sheep pulmonary adenomatosis and therefore results and experiments have been shared, in some occasions, with some of my colleagues. All work of other authors is duly and gladly acknowledged in the text.

This thesis has not been submitted for any other degree.

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Abbreviations:

| | |
|--------------|---------------------------------------|
| AEV | Avian erythroblastosis virus |
| ALV | Avian leukosis virus |
| ALSV | Avian leukosis sarcoma virus |
| APC | Antigen presenting cells |
| BAC | Bronchiolo-alveolar cell carcinoma |
| BLV | Bovine leukaemia virus |
| CA | Capsid protein |
| CHV-1 | Caprine herpesvirus type 1 |
| Ci | Curie |
| CMV | Cytomegalovirus |
| CTL | Cytotoxic T lymphocytes |
| DAB | Diaminobenzidine |
| DEPC | Diethylpyrocarbonate |
| DIG | Digoxigenin |
| dNTP | Deoxynucleoside triphosphate |
| dsDNA | Double stranded DNA |
| EM | Electron microscopy |
| EBV | Epstein-Barr virus |
| ENTV | Enzootic nasal tumour virus |
| ERV | Endogenous retroviruses |
| FACS | Fluorescent activated cell sorter |
| FeLV | Feline leukaemia virus |
| F-MLV | Friend-murine leukaemia virus |
| FV | Foamy viruses |
| GCSF | Granulocyte colony-stimulating factor |
| HERV | Human endogenous retroviruses |
| HFV | Human foamy virus |
| HHV-6 | Human herpes virus type 6 |
| HIV | Human immunodeficiency virus |
| HSV-1 | Herpes simplex virus type 1 |
| HTLV | Human T-cell-lymphotropic virus |
| IAP | Intracisternal A particles |
| IL-2 | Interleukin-2 |
| IL-3 | Interleukin-3 |
| IMC | Infectious molecular clone |
| IN | Integrase |
| JSRV | Jaagsiekte sheep retrovirus |
| kb | Kilo bases |
| LPS | Lipopolysaccharide |
| LTR | Long terminal repeat |
| MA | Matrix |
| MACS | Magnetic activated cell sorting |

| | |
|-------------------------|--|
| MCF | Mink cell focus-forming viruses |
| MDV | Marek's disease virus |
| MHC | Major histocompatibility complex |
| MLV | Murine leukaemia virus |
| M-MuLV | Moloney-murine leukaemia virus |
| MMTV | Mouse mammary tumour virus |
| Mpa | Mega-Pascal |
| MPMV | Mason-Pfizer monkey virus |
| MVV | Maedi-visna virus |
| <i>Mtv</i> | Endogenous mouse mammary tumour viruses |
| NC | Nucleocapsid protein |
| OD₆₀₀ | Optical density at 600 nm wavelength |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Primer binding site |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PO-1-Lu | Langur virus |
| PPT | Poly-purine-tract |
| PR | Protease |
| psi | Pounds per square inch |
| RSV | Rous sarcoma virus |
| RT | Reverse Transcriptase |
| SA-OMVV | South African isolate of maedi-visna virus |
| SDS-PAGE | Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis |
| SFFV | Spleen focus forming virus |
| SMRV | Squirrel monkey retrovirus |
| SRV | Simian retrovirus |
| SSV | Simian sarcoma virus |
| SU | Surface protein |
| TCR | T-cell receptor |
| TM | Transmembrane protein |
| TRE | Tax response element |
| ssRNA | Single stranded RNA |
| SPA | Sheep pulmonary adenomatosis |
| U | Units |
| V | Volts |
| WFB | Washing fluid B |

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ABSTRACT

Sheep pulmonary adenomatosis (SPA) is a naturally occurring contagious lung tumour of sheep which has been associated aetiologically with a chimaeric type D/B retrovirus known as jaagsiekte sheep retrovirus (JSRV). Studies on the aetio-pathogenesis of this disease are extremely valuable in comparative pathology because SPA represents a unique model of a naturally occurring lung cancer.

The role of JSRV in the aetio-pathogenesis of SPA is largely unknown. This is due to several factors which have hampered research for a number of years, such as the lack of a cell culture system for the propagation of JSRV and the lack of reagents and techniques to detect JSRV. In addition, the presence in the sheep genome of 15 to 20 copies of JSRV-related endogenous sequences has impeded investigation at the molecular level.

Studies directed at further understanding the biology of JSRV were undertaken in this thesis. Initially, immunological techniques for the detection of JSRV were developed and employed to establish JSRV sites of replication. In particular, a blocking enzyme-linked immunosorbent assay (B-ELISA) was used to verify the distribution of viral particles in several tissues collected from SPA-affected sheep and unaffected controls. Immunohistochemistry was employed for the detection of the JSRV major capsid protein at the cellular level. The results obtained at the end of these studies pointed to epithelial tumour cells in the lungs of sheep affected by SPA as major sites of viral replication. JSRV viral particles and viral proteins were never detected in non-tumour tissues from SPA-affected sheep or in unaffected control animals.

The relationship between the exogenous JSRV and the highly related endogenous sequences present in the sheep genome (sheep endogenous retroviruses, *enJSRV*) was then determined by RT-PCR coupled to sequencing and restriction enzyme analysis. *EnJSRVs* were found to be transcriptionally active in a wide variety of tissues of

both healthy and SPA-affected sheep. By sequencing a fraction of the *gag* gene of *enJSRVs*, a *ScaI* restriction site was found to be a molecular marker for the exogenous form of JSRV. JSRV provirus was detected in tumour genomic DNA of SPA-affected animals but not in non-tumour tissues of the same animals or in unaffected controls. These results demonstrated that JSRV was a horizontally transmitted virus, specifically associated with SPA, and it was not an endogenous retrovirus reactivated as a downstream event of neoplasia.

Finally, a highly sensitive exogenous-specific hemi-nested PCR was developed utilising primers in the U3 region of the JSRV LTR, where major differences between endogenous and exogenous sequences were found. Employing this test, it was demonstrated that JSRV established a disseminated infection of the lymphoid tissues of sheep affected by SPA, although the epithelial tumour cells are the main sites of viral replication. This finding could be extremely important in future studies aimed at understanding the interaction of JSRV with the host immune system.

In conclusion, the data in this thesis present compelling evidence to specifically associate the exogenous form of JSRV with sheep pulmonary adenomatosis, strengthening the view that this virus has a role in the aetiology of the tumour.

Chapter 1.
Introduction - Review of the Literature

1.1 History of sheep pulmonary adenomatosis.

Sheep pulmonary adenomatosis (SPA) is a contagious and experimentally reproducible lung tumour of sheep, which leads invariably to death of the affected animals.

The disease was recognised for the first time in South Africa in the 19th century as a cause of dyspnoea in sheep being driven, hence the origin of the Afrikaans name “jaagsiekte”, meaning driving (= jaagt) sickness (= ziekte) (Tustin, 1969). Since then, it has been recorded in England (McFadyean, 1894), Germany (Eber, 1899) France (Aynaud, 1926) and in almost all the sheep-rearing countries with the noteworthy exceptions of Australia and New Zealand (Verwoerd *et al*, 1985).

SPA acquired notoriety in the thirties when it was introduced into Iceland by a small number of sheep imported from Germany, along with scrapie, maedi-visna and paratuberculosis (Sigurdsson, 1954). The obvious geographical “barriers” allowed the establishment of the relationship between the imported sheep and the newly emerging diseases.

Sigurdsson (1954) coined the term “slow” to describe this group of diseases because they did not fit either the acute or the chronic pattern of a classical infectious disease. He described slow diseases as diseases with a very long initial period of latency lasting from several months to several years and a regular protracted course after the onset of the clinical signs usually ending with the death of the affected animal.

The observations made in those years have been of great biological importance in the study of the pathogenesis of viral infections and in understanding the complex virus/host relationship .

1.2 Clinical and epidemiological features.

Sheep affected with SPA are likely to show clinical signs only when the tumour volume becomes sufficiently large to interfere with normal lung function. Small areas of tumour may be present without obvious clinical signs. The symptoms which

develop are those of progressive respiratory embarrassment, which reflects the extent of the loss of the normal alveolar function. A pathognomonic sign of SPA is the accumulation of fluid within the respiratory tract (lung fluid). Up to 300 ml of lung fluid can be collected when the rear limbs (and thus the chest) are elevated above the head of the affected sheep ("wheel-barrow test") (Fig. 1.1). The affected sheep usually die within a few weeks once signs become apparent and it is possible to observe a progressive loss of weight and a general decline of the bodily condition of the animal (Sharp and Angus, 1990). Secondary bacterial infections may complicate the clinical picture and accelerate the death of the animal.

SPA is usually seen in sheep that are more than 2 years old with the peak of incidence occurring at 3-4 years of age (Hunter and Munro, 1983). The incubation period in nature is probably several months, based on the observations made by Sigurdsson (1954) in Iceland, and those of early transmission studies by allowing the cohabitation of healthy with diseased sheep (de Kock 1929a, 1929b referred to by Tustin, 1969). However, SPA has occasionally been observed in lambs 3-6 months old (J.M. Sharp, personal communication).

The incidence of the disease in an affected flock has been reported to be usually between 1% and 5% per annum where the disease is endemic (Aliev, 1967; Ceretto and Deiana, 1967; Tustin, 1969; Wandera, 1971; Verwoerd *et al*, 1985; De las Heras, personal communication). However, when SPA was first introduced in Iceland there were losses in some flocks ranging from 50% to 80% within 1 to 2 years (Dungal *et al*, 1938) after which it gradually decreased. An analogous situation has been described in Kenya where losses were initially as high as 30% (Shirlaw, 1959) and dropped subsequently (Wandera, 1967).

In Scotland, prospective longitudinal studies on two flocks have shown that SPA occurred in up to 30% of sheep during the commercial life of the flock and was responsible for over 50% of the mortality (Sharp and Angus, 1990).



Fig 1.1: Nasal discharge of “lung fluid” from a SPA-affected sheep. Up to 300 ml of lung fluid can be collected from a sheep during the “wheel-barrow” test.

1.3 Gross pathology, histopathology and ultrastructure of the tumour.

At necropsy, SPA-affected sheep have lungs that fail to collapse when the thorax is opened and are 2-4 times heavier than normal. Usually it is possible to observe a frothy fluid exuding from the end of the trachea. There is normally a grey firm mass in the cranio-ventral portions of one or both lungs which in some cases can extend to the intermediate and the lateral margins of the posterior lobes (Fig 1.2). When a neoplastic lung is incised a clear or foamy fluid may exude from the airways and the cut surface shows numerous small, slightly-elevated whitish grey nodules in section. There may be necrosis or ulceration involving the tumour or evidence of secondary bacterial infections in affected lungs. Pleural adhesions are often observed. In early cases, solitary tumour nodules may be the only evidence of disease (DeMartini *et al*, 1988; Rosadio *et al*, 1988; Sharp and Angus, 1990).

The lesions are characterised microscopically by the formation of nodular lesions in which the alveoli are lined with cuboidal or columnar cells often forming papillary growths which produce a complicated adenomatous pattern. The foci are supported by a sparse connective tissue stroma. In the majority of cases the presence of large, swollen macrophages in the alveoli contiguous to the focal neoplasia can be detected. Metastases are a major reason for the designation of SPA as a pulmonary carcinoma (OPC) by some authors (Hod *et al*, 1974 and 1977; Perk *et al*, 1974; DeMartini *et al*, 1988; Rosadio *et al*, 1988a and 1988b). The World Health Organisation classifies SPA as a bronchioloalveolar carcinoma (Stunzi *et al*, 1974). In the Awassi sheep of Israel, metastases were found in up to 50% of the cases and were distributed in pulmonary lymph nodes, kidney and skeletal muscle (Nobel *et al*, 1969). In Scotland, South Africa and Peru, the rate of metastasis is reported to be rare and in no more than 10% of SPA cases (Hunter and Munro, 1983; Snyder *et al*, 1983; Verwoerd *et al*, 1985).

Ultrastructural studies have shown the cell types from which the tumour originates to be the type II pneumocytes and the non-ciliated bronchiolar (Clara) cells (Nisbet *et al*, 1970; Perk *et al*, 1971a; Payne and Verwoerd, 1984; DeMartini *et al*, 1987).

The SPA tumour cells maintain the appearance of the cells from which they originated, with microvilli, desmosomes and intracellular lamellar bodies for the type II pneumocytes and cytoplasmic dense bodies for the Clara cells.

Both the type II pneumocytes and the Clara cells are secretory type cells. Type II pneumocytes are extremely important cells regulating the homeostasis of the functional unit of the lung, the alveolus. These cells synthesise, store and secrete the lung surfactant which is a phospholipid-rich substance with the putative function to lower surface tension during expiration (Voelker and Manson, 1989; Chander and Fisher, 1990). The lamellar bodies of the type II pneumocytes are believed to be the site of synthesis and storage of the alveolar surfactant (Massaro, 1981) and, therefore, it has been proposed that the lung fluid secreted by SPA-affected sheep is composed mainly of such material. Type II pneumocytes also have the function of regulation the volume of alveolar fluid by transepithelial solute transport and of re-epithelialization of the alveolar wall after injury (Voelker and Manson, 1989).

The Clara cells of the bronchioles are thought to have as a major function the synthesis, storage and secretion of protein components of the extracellular lining layer of bronchioles (Massaro, 1989).

1.4 Aetiology: contagious nature of the tumour and its experimental transmission.

The contagious nature of SPA has been suspected since the first observations of the disease were made. This was confirmed in 1929 when DeKock (1929a, b) was able to transmit SPA by cohabitation of healthy with diseased sheep.

An elegant experiment performed by Dungal (1946) demonstrated that SPA could be transmitted by aerosol. Dungal made an affected sheep breath through a 20% solution of glycerine in normal saline for thirty minutes and used this solution to inoculate three lambs intratracheally and intrapulmonary. Two of the lambs developed jaagsiekte, one of them showing clinical signs, four months after the experimental

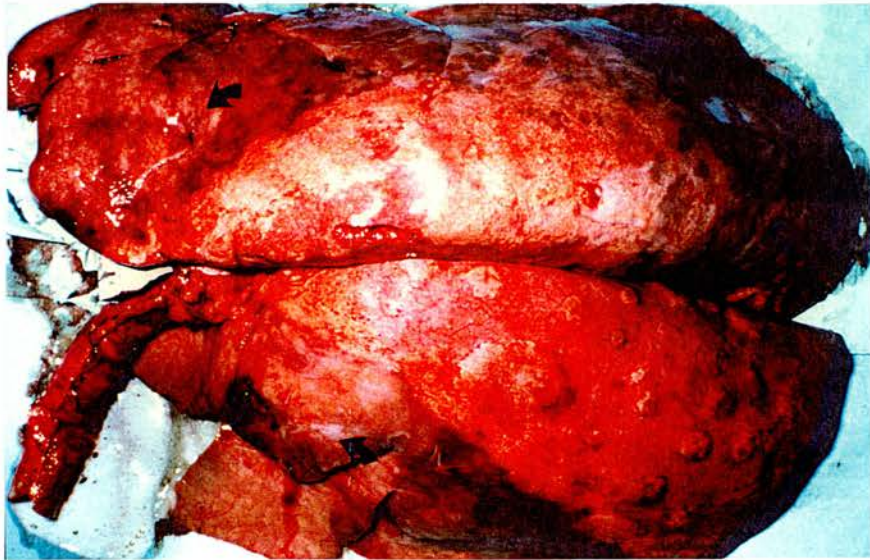


Fig. 1.2: **Sheep lungs with typical SPA lesions.** Note the frothy fluid exuding from the end of the trachea (“lung fluid”). Arrows indicate the neoplastic tissue.

infection. This result was also reproduced with cell-free material after filtering the glycerol-saline solution through a gradacol membrane with pores 0.9 µm in diameter. Several other authors have been able to reproduce the disease by exposing sheep to droplet infection by means of an aerosol spray (Markson and Terlecki, 1964) or by intratracheal or intrapulmonary inoculation of lung tumour homogenates or lung secretions (Sigurdsson, 1958; Enchev 1966, 1968; Cuba-Caparo' *et al*, 1967; Wandera, 1968; Tustin, 1969). All these experiments had a low rate of success and an incubation period of more than 280 days. In contrast, Wandera (1970) had an high success rate (8/10 sheep) using an inoculum more concentrated with respect to the previous experiments.

Transmission of SPA to laboratory animals has failed with the exception of transplantation studies of SPA tumour cells into nude mice (Verwoerd *et al*, 1977; Zimmer *et al*, 1984; Jassim, 1988).

More recent experiments, including those where the incubation period has been dramatically reduced to three-six weeks will be described in 1.9.

1.5 Viruses associated with SPA: herpesviruses.

From the beginning of the century onwards several macro/micro-organisms have been hypothesised to be associated with SPA such as the sheep lungworm *Muellerius capillaris*, Mycoplasma and Chlamydia but none of them was demonstrated to be the aetiological agent of SPA (McFadyean, 1894 and 1920; Taylor, 1938; Mackay, 1966; Tustin, 1969).

An interesting finding was the isolation of herpesviruses from lungs of SPA sheep in geographically distinct locations such as Scotland (Mackay 1969a and 1969b; Martin *et al*, 1979), Yugoslavia (Nevjestic *et al*, 1971), Kenya (Malmquist *et al*, 1972) and South Africa (De Villiers *et al*, 1975). These viruses were all antigenically related and could be differentiated by restriction endonuclease analysis (Scott, 1984). It was remarkable that this agent, designated initially caprine herpesvirus 1 (CHV-1) and subsequently ovine herpesvirus-1 (OHV-1) (Roizman, *et al* 1992), was isolated only

from SPA-tumours, although not from every case and in some studies only in a small minority (Martin *et al*, 1979).

Numerous attempts to reproduce SPA experimentally by the inoculation of sheep with OHV-1 have failed (De Villiers *et al*, 1975; Martin *et al*, 1976 and 1979; Scott, 1984). In addition, the presence of antibodies towards OHV-1 in the sheep population is widespread both in countries where SPA is endemic and in countries where SPA has never been reported (Scott, 1984; De Villiers, 1979); this observation rules out a primary role for this virus in the aetiology of SPA.

The detection of intranuclear inclusion bodies and virions only in pulmonary macrophages suggests that these are the main sites of OHV-1 replication (Scott *et al*, 1984). The increased number of pulmonary macrophages in SPA sheep could explain the isolation of this virus in some of these cases.

1.6 Viruses associated with SPA: retroviruses.

In the early 1970's, virus particles morphologically similar to retroviruses, were detected in SPA tumours by electron microscopy (Perk *et al*, 1971; Bucciarelli, 1973; Hod *et al*, 1977). Since then, circumstantial evidence that a retrovirus may be the aetiological agent of SPA has been accumulating.

An overview of the *Retroviridae* and their association with neoplastic diseases will be given in 1.6.1 to 1.7.3 due to the importance of these viruses in the context of this thesis.

Sections 1.6.1, 1.6.2 and 1.6.3 deal with the structure, genomic organisation, replication cycle and classification of retroviruses. The literature on the fundamental and molecular biology of retroviruses is very extensive and therefore difficult to cite properly. For these sections I refer only to some of the key papers which gave a major contribution to the understanding of this complex group of viruses and to the following reviews: Teich, 1984; Weiss, 1984, 1993 and 1996a; Stoye and Coffin, 1985; Coffin, 1990a, 1990b, 1991 and 1992; Luciw and Leung, 1992; Coffin *et al*, 1995; Weiss and Tailor, 1995; Kupiec and Sonigo, 1996.

1.6.1 Structure and genomic organisation of retroviruses.

The *Retroviridae* comprises a large family of RNA viruses of vertebrates extremely widespread in nature. The initial observations leading to the discovery of this class of viruses were all related to tumour-associated viruses (Ellerman and Bang, 1908; Rous, 1911a and 1911b; Staff of Roscoe B. Jackson Memorial Laboratory, 1933; Bittner, 1936; Gross, 1951), however, retroviruses are indeed capable of inducing many different diseases such as immunodeficiencies, neuropathies, pneumonia, bone and joint diseases as well as lifelong viremia in the absence of any obvious clinical symptom. Despite the wide variety of species infected, routes of transmission and disease manifestation, retroviruses are all quite similar in terms of virion structure, genomic organisation and replication cycle.

Virions are spherical with a diameter of about 100 nm. The viral particles are surrounded by an envelope often with prominent spikes composed of virus-encoded glycoproteins (Rifkin and Compans, 1971). The internal core is a spherical to rod-shaped capsid and contains the viral genome. The genome consists of two identical molecules of positive sense single-stranded RNA (7 - 10 kb in length) (Beemon *et al*, 1974; Billeter *et al*, 1974; Quade *et al*, 1974), held together by hydrogen bonds, with a structure reminiscent of cellular mRNA with a cap structure at the 5' end and polyadenylated at the 3' end (Kung *et al*, 1975; Bender *et al*, 1976, 1978). Each RNA molecule is associated with a specific molecule of tRNA that is base-paired to a region (termed the primer binding site, PBS) near the 5' end of the RNA (Taylor and Illmensee, 1975). In all retroviruses there are four main genes encoding for the virion proteins invariably in the same order: 5'-*gag-pro-pol-env*-3' (Wang *et al*, 1976a and 1976b) (Fig 1.3). The *gag* gene encodes for at least three proteins: the MA (matrix) protein, the major capsid protein (CA) and the NC (nucleocapsid) protein. The *pro* gene encodes for a protease (PR) while the *pol* gene is responsible for the synthesis of the reverse transcriptase (RT) and of the integrase (IN). The *env* gene contains the information necessary for the synthesis of the envelope protein, the SU (surface) and the TM (transmembrane) glycoproteins.

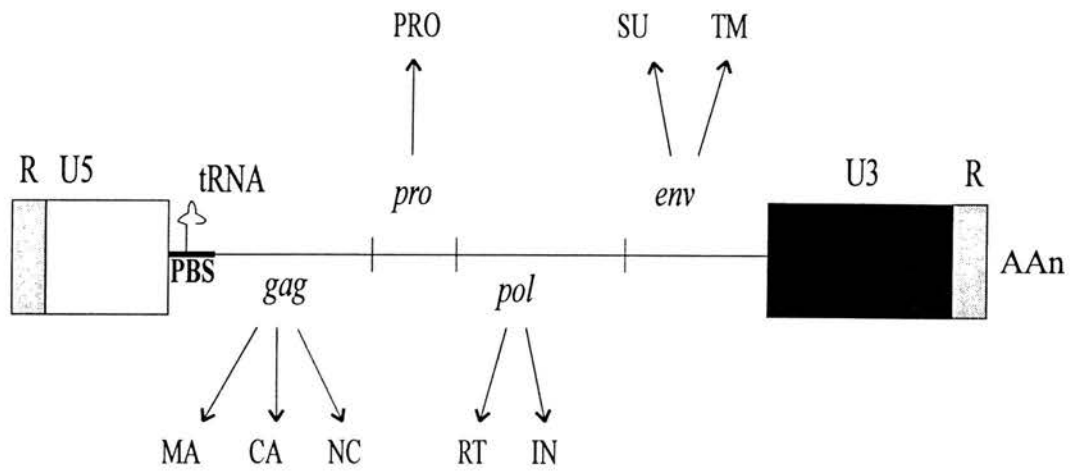


Fig. 1.3: **Features of the retrovirus genome.** The proteins encoded from each gene are also indicated (see page viii for abbreviations used).

Figure 1.4 shows a schematic diagram of the structure of a retrovirus. All retroviruses contains non-coding regions at the ends of the genome which are essential for the replicative strategy of these viruses (see below). These regions are the U5, a region unique for the 5' end of the viral genome, the U3, unique for the 3' end of the genome and R, repeated at both ends of the viral genome.

Some retroviruses ("complex" retroviruses; see 1.6.3) encode non virion proteins which are important for the regulation of expression. Others have the ability to incorporate fragments of certain cellular genes (known as *c-onc* or proto-oncogenes) into their genome and to alter their structure and expression in ways that enable them to directly transform a normal cell into a malignant one (see 1.7.1) (Hanafusa *et al*, 1977; Stehelin *et al*, 1976; Oskarsson *et al*, 1980; Blair *et al*, 1981).

1.6.2 Retroviral life cycle.

The most disparate biological characteristics of retroviruses, such as their oncogenic potential or the ability of these viruses to establish a latent infection, can be fully appreciated when the retroviral life cycle is analysed.

Two major events characterise the life cycle of retroviruses: the reverse transcription of the single stranded RNA genome into double stranded DNA and the integration of this DNA copy into the cell genome.

The retroviral replication cycle can be divided into the following steps: viral entry, synthesis of negative strand DNA, synthesis of positive strand DNA, integration, transcription, translation, assembly and budding.

- ***Viral entry***

The replication cycle starts with binding of the virion to specific receptors at the cell surface. A number of retroviral receptors have been identified. These include the CD4 cell surface protein for HIV (Dalglish *et al*, 1984; Klatzmann *et al*, 1984; Madden *et al*, 1986); a cationic amino acid transporter for murine ecotropic leukaemia virus (Kim *et al*, 1991; Wang *et al*, 1991); a phosphate permease for gibbon ape leukaemia virus (Johann *et al*, 1992), and the avian sarcoma virus

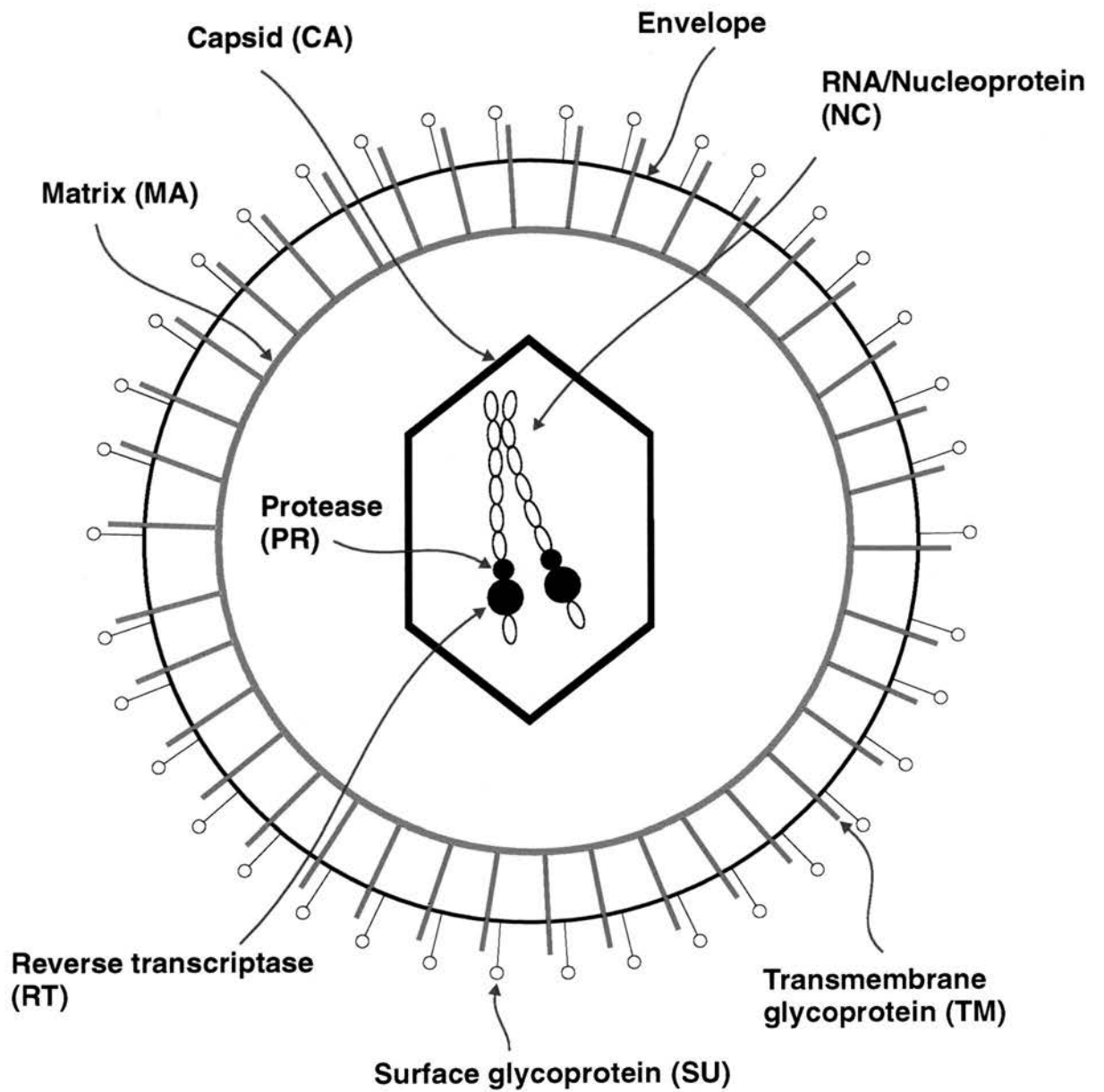


Fig 1.4: Schematic diagram of the retrovirus virion (modified from Coffin, 1990a).

subgroup A receptor which is a molecule related to the low density lipoprotein receptor (Bates *et al*, 1993). Thus, retroviruses appear to take advantage of host-encoded cell-surface proteins that play important roles in cellular function and metabolism. The use of different receptors by related viruses helps to classify some retroviruses into cross-neutralisation subgroups (e.g. avian leukosis viruses and murine type C retroviruses - see 1.6.3).

The SU glycoprotein mediates binding to the cellular receptor. Conformational changes of the cellular receptor and of the envelope proteins follow and lead to the exposure of hydrophobic domains of the TM protein that are believed to influence viral fusion (Weiss, 1993 and 1995).

Usually a retroviral receptor is composed by a single molecule. However, for HIV it has been demonstrated that the CD4 molecule is not sufficient for entry but requires fusion cofactors on the target cell which have been identified as the chemokine receptor CC-CRK-5 (Alkhatib *et al*, 1996; Deng *et al*, 1996; Dragic *et al*, 1996) and a related protein known as “fusin” (Feng *et al*, 1996).

After binding and fusion there is viral penetration into the cell by mechanisms not completely understood. Once inside the cell, viral uncoating takes place. The nucleocapsid (comprising the diploid genome, primer tRNA, reverse transcriptase, integrase and at least some capsid proteins) is responsible for the next steps of replication which involves a DNA intermediate (the provirus) initially hypothesised by Temin (1963).

- ***Synthesis of negative-strand DNA***

The reverse transcriptase associated with the nucleocapsid synthesises double-stranded DNA using the viral genomic RNA as a template (Baltimore, 1970; Temin and Mizutani, 1970). In this process two steps can be identified: synthesis of negative strand DNA (minus DNA, complementary to the positive RNA genome) and synthesis of positive strand DNA (plus DNA, complementary to the minus DNA).

Synthesis of minus DNA is primed by the tRNA coupled to the PBS at the 5' end of the retroviral genome and proceeds towards the U5 and R (Fig. 1.5 [a]) (Verma *et al*, 1971; Dahlberg *et al*, 1974; Harada *et al*, 1975; Taylor and Illmensee, 1975). The

newly synthesised DNA (called at this stage negative strand “strong-stop” DNA [Coffin and Haseltine, 1977]) “jumps” to the 3’ end of the genome (Fig. 1.5 [b]) driven by the strong affinity of the RT for its template. This jump is made possible by the redundancy of the R region at the 5’ and 3’ ends of the viral genome (Gilboa *et al*, 1979) and by the RNase (H) activity of the RT which removes the newly copied RNA (at the 5’ end), leaving the DNA free to pair with the R sequence at the other end (the 3’ end)(Mölling *et al*, 1971). The transfer of the strong-stop DNA can occur both to the same strand on which synthesis started or it can switch to the other strand of the bimolecular RNA genome (Panganiban and Fiore, 1988). This jump can lead to viral recombination between two different retroviruses co-infecting the same cell (Coffin, 1979). Once the jump has occurred, the synthesis of the negative strand proceeds to the 5’ end of the template, which is now the 5’ end of the PBS because R and U5 have been removed by RNase H (Fig 1.5[c]).

- ***Synthesis of positive-strand DNA***

The RT now has to copy the minus-strand DNA to form a double-stranded molecule. There is therefore the necessity for a second primer which is provided by a purine-rich sequence (polypurine tract or PPT) situated immediately 5’ of the 3’ LTR. The RNA sequence of the PPT is resistant to the retroviral RNase H activity, which degrades the RNA genome after minus strand cDNA synthesis (Fig. 1.5[d]) (Varmus *et al*, 1978; Finston and Champoux, 1984; Ratnayake and Champoux 1987 and 1989; Huber and Richardson, 1990; Luo *et al*, 1990; Pullen *et al*, 1993). In some retroviruses, it has been shown that purine-rich sequences in the genome resembling the PPT can provide additional initiation sites (Finston and Champoux, 1984; Ratnayake and Champoux, 1987). In the avian retroviruses the plus strand DNA contains multiple discontinuities suggesting a multiple priming mechanism (Boone and Skalka, 1981a and 1981b; Kung *et al*, 1981; Hsu and Taylor, 1982) while MMTV seems to utilise only one PPT in a single priming event (Kung *et al*, 1981). In lentiviruses and spumaviruses the PPT is duplicated at the end of the *pol* open reading frame suggesting a dual-priming model for these viruses (Sonigo *et al*, 1985; Kupiec *et al*, 1988).

After initiation, elongation of the positive strand DNA is carried out by the RT to the 5' end of the minus-strand including the PBS (Fig. 1.5[d]). The newly formed positive DNA (positive-strand, strong-stop) needs another 'jump' to permit complete synthesis. The PBS of the 3' end of the positive strand is in this case the redundant sequence which binds the PBS at the 3' end of the negative strand (of the same molecule) (Fig.1.5[e]) (Gilboa *et al*, 1979). Completion of the full length, double stranded DNA, can now be accomplished by the synthesis of each strand until completion. This model of replication seems to be accepted for most retroviruses. However, lentiviruses and spumaviruses seem not necessarily to fit this model due to the dual PPT priming (Li *et al*, 1993; Kupiec and Sonigo, 1996) .

The linear dsDNA thus contains at both ends identical long terminal repeats (LTRs) formed in order by U3, R and U5 (Fig. 1.5[f]) (Hughes *et al*, 1978). Along with the linear double-stranded DNA two additional forms appear, the covalently closed DNA circles with one or two LTRs; these are thought to be a by-product of viral DNA synthesis (Brown *et al*, 1987).

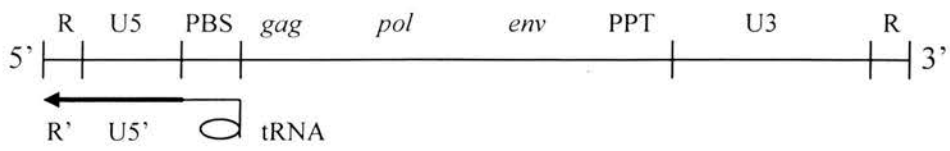
The LTRs, and in particular U3, contain a transcriptional promoter. Also contained within U3 are the enhancer elements which potentiate the transcriptional activity of the adjacent promoter (Khoury and Gruss, 1983) by binding specific cellular proteins (transcription factors) (Herr and Clarke, 1986; Fromental *et al*, 1988).

The RT lacks a 3'→5' exonuclease proof-reading activity and therefore it has no means of correcting occasional errors that arise during polymerisation. This is a major cause of the high frequency of retroviral mutation (0.05-1 mutations per genome per replication cycle) (Preston and Dougherty, 1996) leading to the generation of 'quasispecies'. The RT of some viruses seem to be more error prone than others for example HIV-RT has a misincorporation rate higher than MLV-RT or ALSV-RT (Preston *et al*, 1988; Roberts *et al*, 1988).

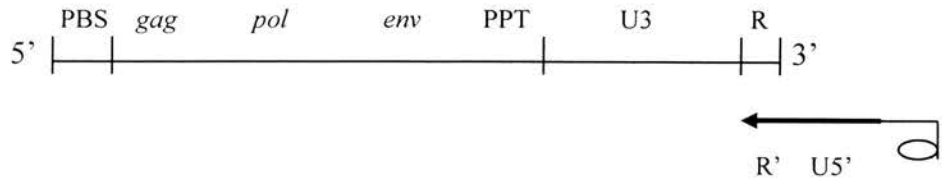
- ***Integration of the provirus into the cell DNA***

The viral DNA is then transferred to the nucleus and integrated into the chromosomal DNA of the host to form a *provirus* by a mechanism involving the viral IN protein. Integration appears to be a prerequisite for replication of at least the great majority of

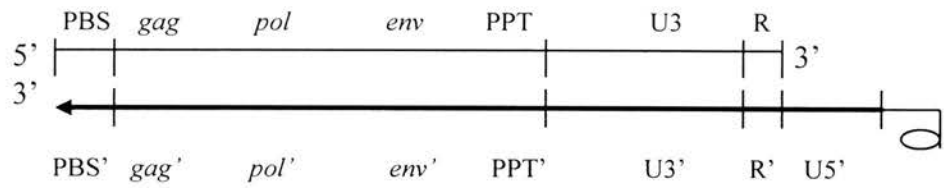
a)



b)



c)



continues next page

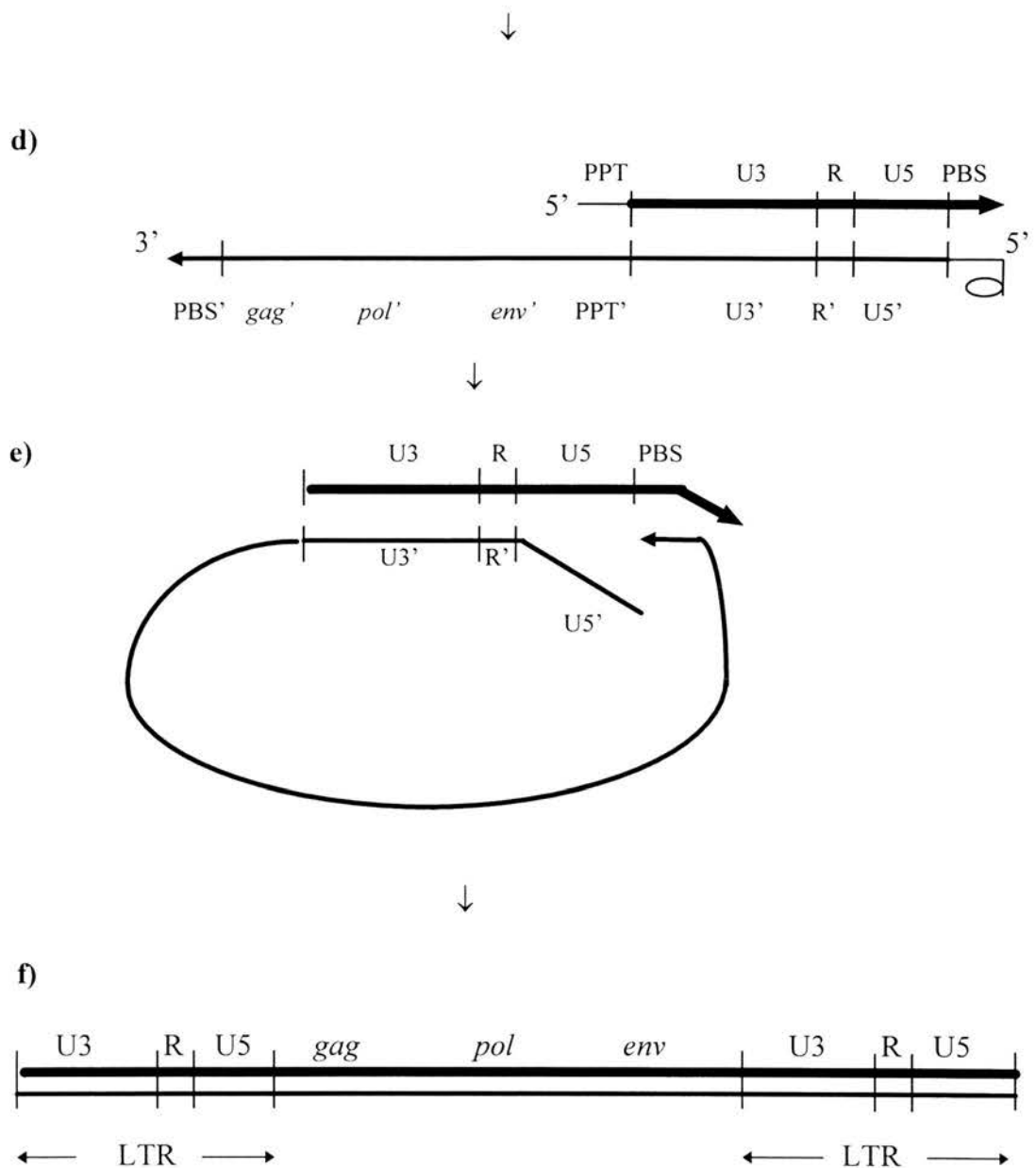


Fig. 1.5: **Viral DNA synthesis.** Thin lines represent RNA, medium lines represent negative-strand DNA, and thick lines represent positive strand DNA. (a-c): synthesis of negative strand DNA; (a), tRNA priming; (b), first RT jump; (c), completion of minus strand DNA. (d-f): synthesis of positive strand DNA; (d): PPT priming; (e): second RT jump; (f): complete provirus (modified from Coffin 1990a).

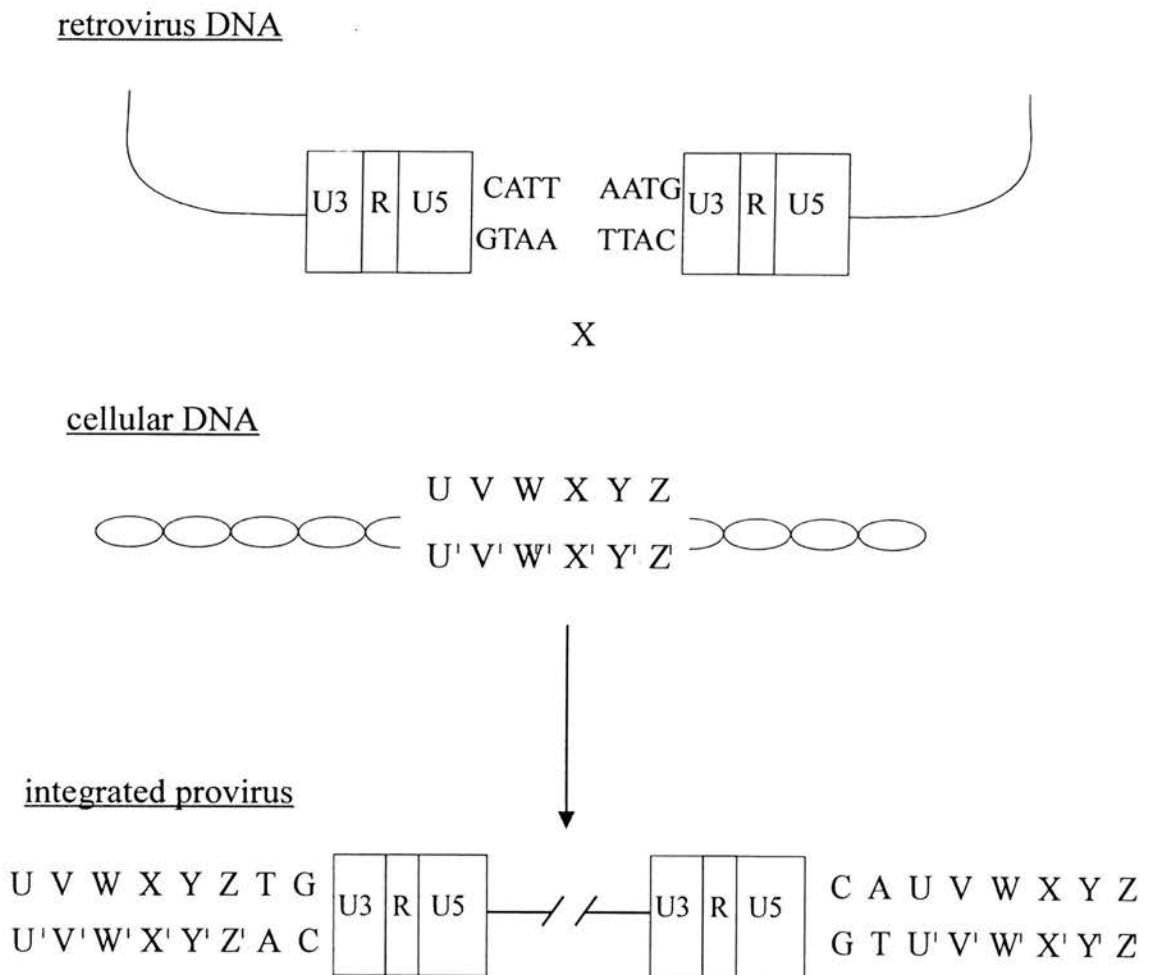


Fig. 1.6: **Diagram representing some features of retrovirus integration.** The top part shows the juxtaposed ends of unintegrated viral DNA. The middle portion shows the target cell DNA with the integration site; the integrated provirus is shown below. Note the loss of two base pairs from each end of the provirus and the duplication of the cellular base pairs (redrawn from Coffin, 1990a).

retroviruses although it is very difficult to detect integrated proviruses of maedi-visna virus and spumaviruses (Harris *et al*, 1981; Kupiec *et al*, 1988). Retroviral integration is essentially a random event (Brown, 1990). However, DNaseI-hypersensitive sites are used for integration at a frequency much greater than expected (Vijaya *et al*, 1986; Rohdewohld *et al*, 1987) and integration *in vivo* occurs preferentially in cells that are actively replicating their DNA (Varmus *et al*, 1977; Humpries *et al*, 1981). It has also been reported that transcriptionally active regions of the genome are preferred as sites for integration (Scherdin *et al*, 1990).

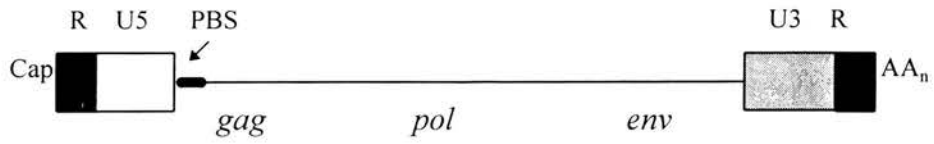
The exact mechanisms of integration are not completely understood, however some steps have consistently been found with all retroviruses (Fig. 1.6). The linear double-stranded DNA is the integrated form. Two bases at both 3' termini of the provirus are removed by the IN protein, leaving recessed 3' ends. A staggered cut is then made in the target DNA with 4-6 bases of overhanging 5' ends which are then ligated to the 3' ends of the provirus. A cellular repair system then removes the two mismatched bases at the 5' ends and fills in the resulting gap and ligates the remaining ends. In this way the cellular DNA flanking the proviral genome is not grossly changed except for the duplication of a short sequence (4-6 base pairs) immediately adjoining the viral DNA (Fig. 1.6). Once integrated the provirus appears to be incapable of transposition (Brown, 1990).

- **Transcription**

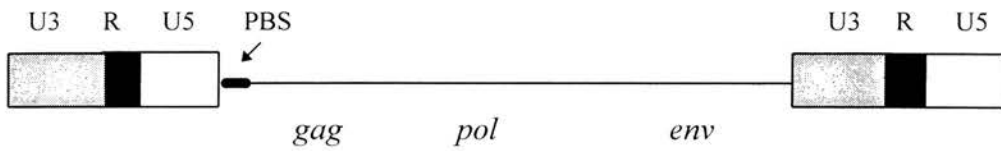
Once integrated, the provirus uses the cellular machinery of transcription for its expression. The 5' LTR provides the signals which are then recognised by the cellular transcriptional machinery.

As indicated earlier U3 contains the viral promoter and enhancer sequences. Promoters are DNA sequence elements that contain binding sites for various protein factors that initiate and regulate transcription (Lewin, 1990). All known retroviruses, in common with many viral and cellular genes, have a TATA box in the U3. In the cellular model of transcription the TATA box binds a cellular factor (TATA-binding protein, TBP) which interacts with other cellular factors and RNA polymerase II to initiate synthesis of RNA in a template dependent manner (Lewin, 1990; Greenblatt,

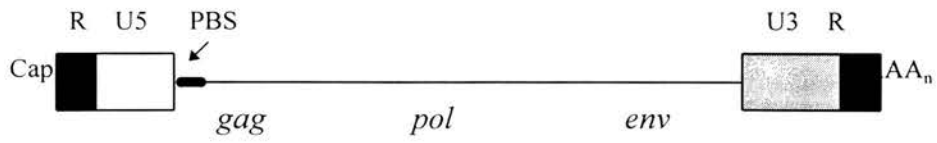
A)



B)



C)



D)

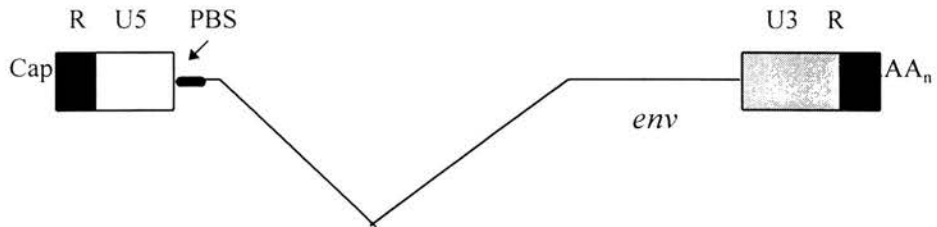


Fig. 1.7: **Nucleic acid species during the replication cycle of a “simple” retrovirus.** A: viral genome; B: provirus; C: full length transcript; D: single spliced transcript.

1991). The viral enhancers are another class of *cis*-acting sequences that regulate viral transcription indirectly by enhancing or activating transcription from the adjacent promoter. The enhancers are usually located 100 - 200 bp upstream of the TATA box (Athas *et al*, 1994) (for their influence on retroviral pathogenesis see 1.7.2).

Provirus contains a single transcriptional unit. All genomic and mRNA species derive from a primary transcript that starts at the 5' U3-R junction (where the capping group is placed) and proceeds through the 3' LTR, with the final 3' end determined at the end of R by cleavage and polyadenylation (Fig. 1.7[c]). All retroviruses use full-length transcripts for their genomes and for the mRNA encoding the *gag*, *pro* and *pol* products. A spliced mRNA consisting of the 5' end of the genome, the *env* gene and the U3 and R regions is produced for translation of the precursor of the envelope proteins (Fig. 1.7[d]) (Hayward, 1977; Weiss *et al*, 1977). Retroviruses which use only these two transcripts are generally addressed as "simple" retroviruses in contrast to "complex" retroviruses (see 1.6.3) where other subgenomic spliced transcripts encoding for regulatory proteins such as the transactivating *tat* or *tax* in HIV and HTLV are observed (Fig 1.8). The subgenomic messages (*env* included) are made by splicing a leader sequence at the 5' end of viral RNA to an acceptor sequence within the viral genome; in this way all spliced viral messages utilise a common splice donor and share 5' and 3' ends.

A distinct replication pathway has been recently discovered for foamy viruses (HFV; see 1.6.2) where the Pol protein is translated from a spliced mRNA and lacks Gag domains. Interestingly, HFV infectious particles contain double-stranded DNA similar in size to full length provirus, suggesting that reverse transcription takes place in viral particles before a new round of infection (Yu *et al*, 1996).

- **Translation**

The translation of retroviral mRNA follows the standard cellular "scanning" model in which ribosomal subunits bind initially to the capping group and move along the RNA until an AUG initiation codon (Petersen and Hackett, 1985).

In all retroviruses the *gag*, *pro*, and *pol* genes can be viewed as forming a single translation unit. This is probably to assure the correct ratio of the various proteins and to provide that the Pro and Pol proteins are strictly associated with the structural proteins to be incorporated into virions. For *pro* and *pol*, translation is made possible by ribosomal frameshifting or by suppression of the stop-codons at the *gag-pro* and/or the *pro-pol* boundaries of translational termination signals (Jack and Varmus, 1985; Coffin 1990a).

The Env precursor product is synthesised on polyribosomes associated with the rough endoplasmic reticulum and then is cleaved by cellular enzymes, at the Golgi apparatus level, into the SU and TM which will remain associated with one another and anchor at the cell surface.

- ***Virion assembly and budding***

This process is a poorly understood aspect of retroviral replication.

It is assumed that assembly involves the interaction of an RNA motif (s), 'ψ' ('psi'), situated at the 5' end of the genome, with the gag precursor protein and in particular with the NC. The CA interacts with the CA region of other precursor molecules and the MA precursors interact with the cell membrane acquiring the envelope of the virion. In type B and D retroviruses (see 1.6.3) the nucleocapsid structure is formed before they acquire the envelope while in the other retroviruses the virion assembles while budding and acquiring the envelope (Bolognesi *et al*, 1978; Rein *et al*, 1994). Following assembly, around the point of budding, viral particles undergo "maturation" under the action of PR protein which cleaves the precursor proteins into their individual components.

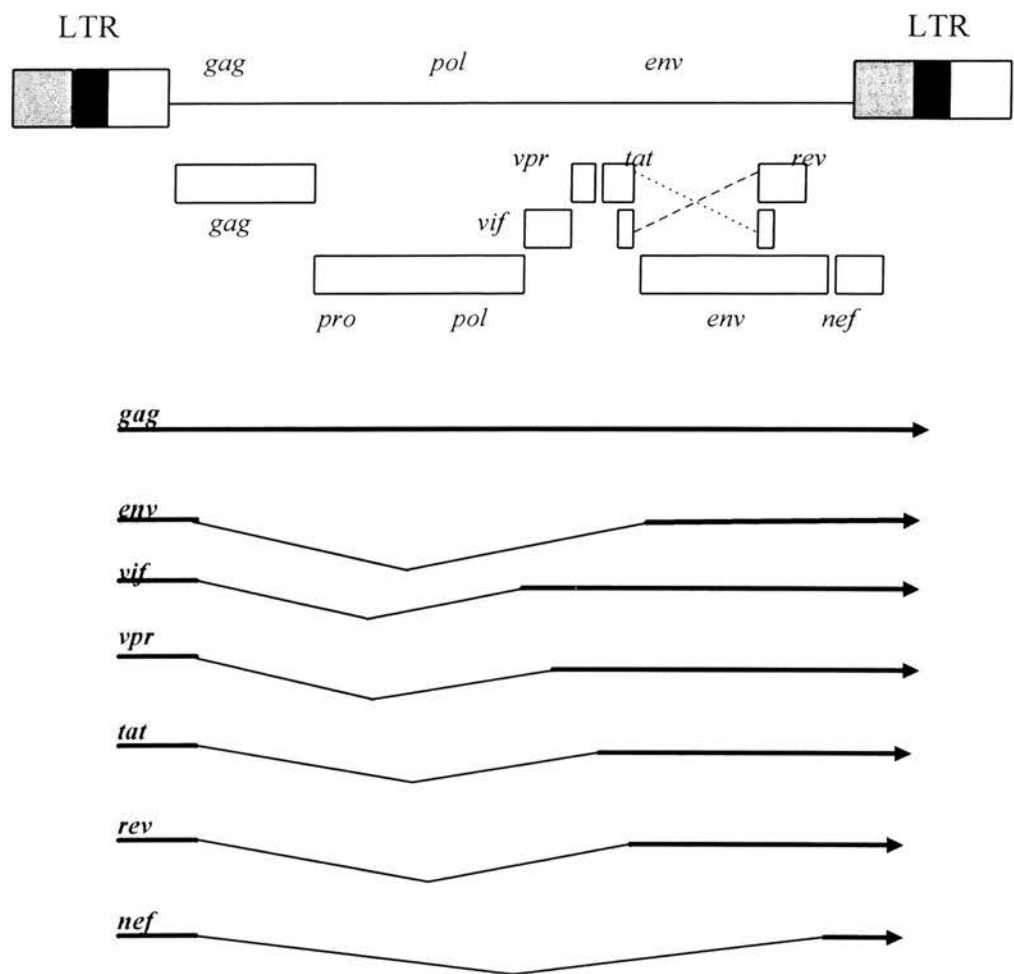


Fig. 1.8: **Genomic organisation of HIV** as an example of “complex” retrovirus. Seven different mRNA species are visible. Note that all spliced viral messages utilise a common splice donor and share 5' and 3' ends (modified from Coffin *et al*, 1995).

1.6.3 Classification of retroviruses.

The family of the *Retroviridae* comprises all the RNA viruses with a RNA-dependent DNA polymerase (Fenner, 1975). Different parameters have been used over the years to classify the members of this family.

Traditionally, retroviruses have been divided, on the basis of their pathogenicity, into three subfamilies: (i) *Oncovirinae*, including all the oncogenic viruses and many related non-oncogenic viruses; (ii) *Lentivirinae*, including the “slow” viruses like maedi-visna virus and (iii) *Spumavirinae*, (spuma = foamy) which induce vacuolisation *in vitro* but are not associated with any disease *in vivo*.

Based on morphology, retroviruses, have been further classified into type-A, -B, -C and -D (Teich, 1984; Coffin, 1992) (Fig. 1.9). Type-A particles are defined as an intracellular form only; they have a diameter of 60-90 nm and are spherical with an electron-lucent centre and a double mantle. A-type particles are not infectious. They are the intracytoplasmatic forms of type-B and -D (see below). Similar particles are seen in some rodent cell lines and are the product of endogenous (see below) elements known as IAP (intracisternal A particles) which contain two LTRs, *gag* and *pol* but their *env* is defective in all forms. IAPs with associated RT activity, contain polyadenylated RNA and accumulate in the cisternae of the endoplasmic reticulum but they have no extracellular phase and are not infectious (Kuff and Lueders, 1988). IAP-related envelope encoding proviral elements have also been demonstrated in mice (Reuss and Schaller, 1991; Reuss *et al*, 1996).

Type-B particles are the extracellular forms of MMTV. Assembly occurs within the cytoplasm as type-A particles prior to budding. Mature extracellular mature particles have an eccentric condensed core and prominent surface spikes. The size of the particles is about 125 nm in diameter.

Type-C particles differs from type-B because the assembly occurs at the inner surface of the cell membrane at the same time as budding and therefore no immature forms are observed in the cytoplasm. An electron-dense crescent-shaped form is the first distinct virion structure to be seen at the electron microscope level. As viral

maturation proceeds it is possible to observe a spherical core with an electron lucent centre surrounded by the plasma membrane which will form the envelope. The mature extracellular forms (80-110 nm diameter) have a centrally located electron-dense core with barely visible surface projections (Bouillant and Becher, 1984).

Type-D particles are associated with both intracellular and extracellular particles. The intracellular particles are 60-95 nm in diameter and ring-shaped. The extracellular particles measure 100-120 nm in diameter and contain a characteristic cylindrical core with surface projections which are less prominent than type B-particles.

This classification is still used to a certain extent although some of the viruses described in recent years, such as human immunodeficiency virus (HIV) or human T cell leukaemia virus (HTLV), do not fall completely into any of these categories.

On the basis of their lifestyle retroviruses can be divided into “exogenous”, horizontally transmitted viruses, and “endogenous” (see 1.6.4), vertically transmitted from parent to offspring as a provirus integrated into the germline.

Another division is “simple” and “complex” retroviruses, which refers to the genomic organisation. Simple retroviruses have only *gag*, *pol*, and *env* genes while complex retroviruses have additional genes that are important for the regulation of expression (Cullen, 1992; Felber and Pavlakis, 1993; Peterlin, 1995). Complex retroviruses include HIV, HTLV, BLV, spumaviruses and others. For example, HIV has three regulatory proteins: *tat*, *rev* and *nef* which play a positive role in viral expression.

The classification adopted from the International Committee for the Taxonomy of Viruses is based on nucleotide sequence relationship and genome structure of retroviruses but also takes into consideration previous criteria. Retroviruses are thus divided into the following 7 genera:

a) Mammalian type B oncovirus group

- prototype: MMTV

This group includes only mouse mammary tumour virus (MMTV), the aetiological agent of mammary carcinoma in mice.

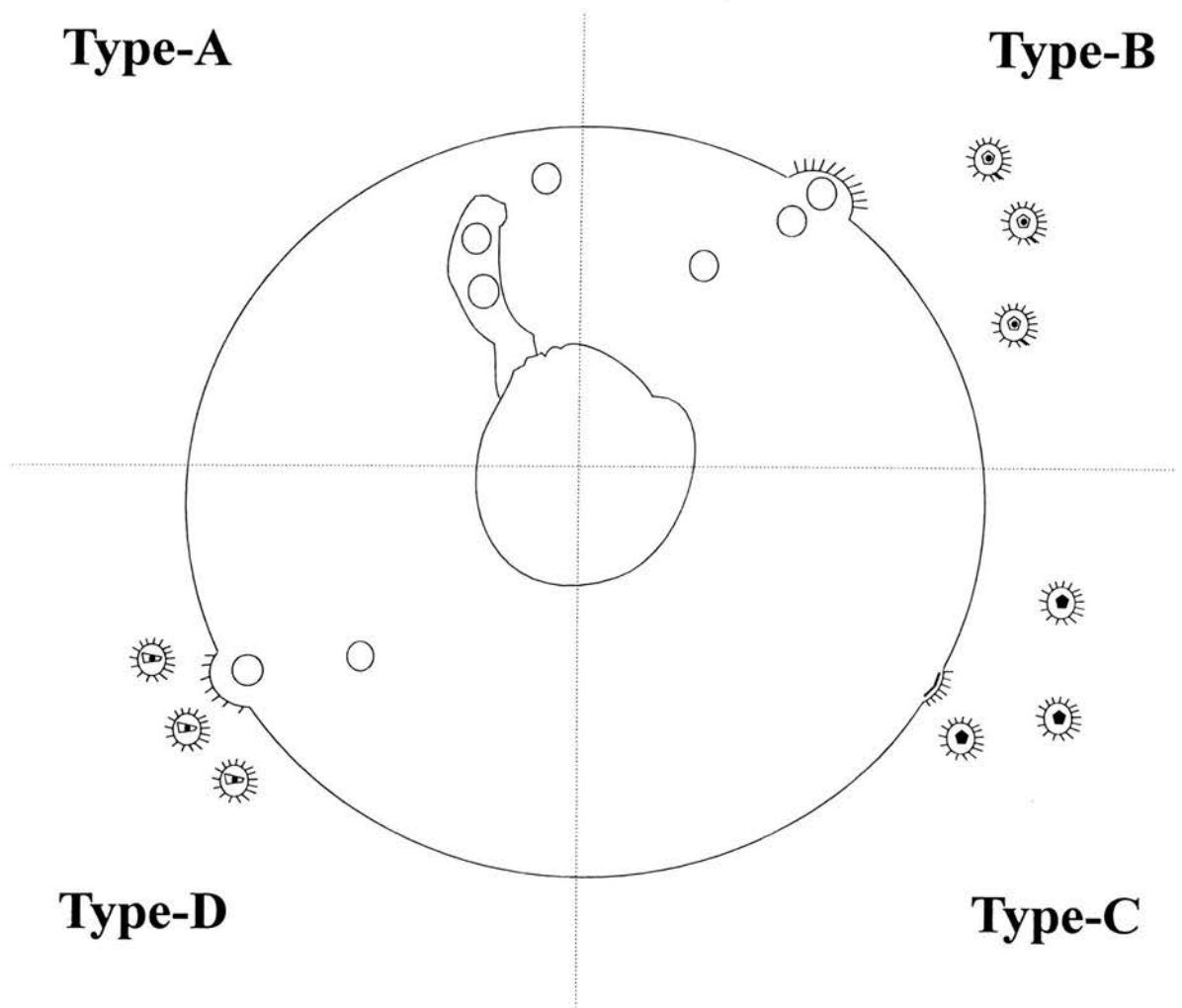


Fig. 1.9: **Morphologic classification of retroviruses into type A → D.** Type A particles are shown as rings and are exclusively intracellular. Type B and D retroviruses assemble into the cytoplasm in forms similar to type A. In type C retroviruses the assembly occurs at the inner surface of the cell membrane at the same time as budding.

Susceptible strains, such as C3H, acquire the virus through milk-born infection and can be freed of this route of infection by foster-nursing on non-viremic mothers, while other strains inherit an endogenous copy of the provirus that is activated in the mammary gland *in situ* and cannot be freed of the virus by foster-nursing. For example, GR mice appear to activate one endogenous provirus (*Mtv-2*) that is shed into milk and can infect other strains as an exogenous, milk born virus (Bentvelzen and Hilgers, 1980; Michalides *et al*, 1983). Although endogenous MMTV proviruses are present in the germ line of all inbred mice and there are multiple proviral sequences found at different chromosomal locations in different mouse strains, the majority of these do not produce virus. At least 10 different exogenous and greater than 30 endogenous MMTVs have been identified (Kozak *et al*, 1987).

MMTV is associated primarily with mammary carcinoma (Bentvelzen and Hilgers, 1980; Michalides *et al*, 1983) but variants of the virus have been shown to induce T cell lymphoma (Michalides *et al*, 1982 and 1985; Ball *et al*, 1988; Hsu *et al*, 1988) and be associated with kidney adenocarcinomas (Garcia *et al*, 1986; Wellinger *et al*, 1986). As the name of the genus indicates, the morphology is of type B with a simple genomic organisation.

Among the retroviruses, MMTV has recently gained a particular interest for its interaction with the host immune system through the expression of a “superantigen” (Sag) (Acha-Orbea and MacDonald, 1993; Held *et al*, 1994; Huber, 1995). A superantigen differs from a conventional antigen because it crosslinks directly the major histocompatibility complex (MHC) class II and the V β domain of the T-cell receptor (TCR) outside the peptide binding groove causing strong activation of the responsive T cells. A Sag is not actually “recognised” by a T cell clone expressing a particular TCR but elicits a multiclonal response, which is restricted to T cells expressing a certain TCR V β chain. In the mouse there are about 20 different TCR V β , therefore a superantigen stimulates about 5% of the total T cells; approximately 10⁴ fold more T cells than those ones activated by a classical peptide/MHC complex. Paradoxically, Sag activation is a requisite step in the MMTV infection pathway (Acha-Orbea and MacDonald, 1993; Held *et al*, 1994; Huber, 1995).

b) Mammalian type C retrovirus group (MLV-related viruses)

- prototype: murine leukaemia virus (MLV)

This group includes a large number of exogenous and endogenous viruses isolated from many groups of mammals, including rodents (murine leukaemia viruses - MLV), carnivores (feline leukaemia virus - FeLV), primates (gibbon ape leukaemia virus), as well as some exogenous viruses of birds (Avian reticuloendotheliosis virus) and reptiles (viper retrovirus).

The murine viruses can be further designated by the species distribution of their receptors; “ecotropic” viruses replicate only in mouse cells, they are found both as endogenous and as exogenous viruses; “xenotropic” viruses are found as endogenous viruses in some mice strains and replicate only in non-murine cells; “polytropic” and “amphotropic” viruses use different receptors found in both mouse and non-murine species. Polytropic viruses (also termed MCF MLVs, for mink cell focus-forming MLVs) are exogenous viruses arising *de novo* as recombinants between infectious ecotropic viruses and endogenous polytropic MuLV-related sequences. They are typically isolated from high leukaemic strains of mice which chronically express high levels of ecotropic virus, such as AKR mice, or from mice inoculated with various exogenous ecotropic MuLVs. Amphotropic viruses have been isolated exclusively from wild mice in southern California (Kozak and Ruscetti, 1992).

Recently, on the basis of sequence analysis of *pro* and *pol* it has been proposed to divide this group into two subgroups (Tristem *et al*, 1996). All of the exogenous MuLV-related retroviruses which have been isolated to date, as well as several endogenous viruses (e.g. baboon endogenous virus) fall into the first subgroup, whereas the second group includes only endogenous retroviruses included HERV-E (see 1.6.4).

The genomic organisation of this group of viruses is that one of “simple” retroviruses.

c) Type D retrovirus group

- prototype: Mason-Pfizer monkey virus (Simian retrovirus type 3 - SRV-3)

This group of viruses comprises exogenous viruses of asian macaques (simian retroviruses, SRV) and endogenous viruses in the Old World spectacled langur (PO-I-Lu) and the New World squirrel monkey (squirrel monkey retrovirus, SMRV). A type D virus isolated from a human lymphoblastoid line, very closely related to SMRV, is referred to as SMRV-H (Oda *et al*, 1988).

Only the exogenous type D viruses of macaques are pathogenic and are now classified into five distinct neutralisation serotypes SRV-1→5 (Gardner *et al*, 1994). SRVs cause a profound depletion of T and B cells and a fatal immunodeficiency syndrome in macaques which should not be confused with the SIV induced immunosuppression in macaques (Letvin *et al*, 1985).

SRVs are not oncogenic although the original type D retrovirus (Mason-Pfizer monkey virus -SRV-3) was isolated from a mammary carcinoma of a rhesus monkey (Chopra and Mason, 1970). SRV-2 is associated with a proliferative disorder called retroperitoneal fibromatosis (RF) which has some features in common with Kaposi's sarcoma (Bryant *et al*, 1986).

They have a type-D morphology and the genetic organisation is that one of simple retroviruses with no accessory genes or oncogenes (see 1.7).

d) Avian type C retrovirus group (ALV-related viruses)

- prototype: avian leukosis virus (ALV)

This group contains the widespread exogenous and closely related endogenous viruses found in chickens and other birds (Payne, 1992). Viruses in this group are associated with a number of neoplastic diseases and some other diseases such as wasting and osteopetrosis. They are further classified according to the host range into seven subgroups (A → J). A to D are exogenous viruses of chickens (A and B common strains, C and D rare ones); ubiquitous endogenous leukosis viruses of chickens belong to subgroup E. Endogenous viruses of pheasant are included in subgroups F (isolated from the ring-necked pheasant) and G (isolated from the Golden pheasant). Endogenous viruses of subgroup H have been isolated from the Hungarian partridge and that of subgroup I from Gambel's quail. A recently isolated

strain of an exogenous ALV of chickens (strain HPRS-103) has been classified into the new subgroup J (Payne *et al*, 1992).

Avian type C retroviruses have a simple genomic organisation. Many oncogene-containing members (e.g. Rous sarcoma virus) have been isolated in this genus.

e) Foamy virus group

- prototype: human foamy virus

Foamy viruses (FV) were originally isolated as agents that cause vacuolation in cell culture. This group comprises exogenous viruses of different animal species including monkeys, cats, cattle and humans. The presence of exogenous human foamy viruses has been recently questioned after the finding that the sequence of the 'human' isolate is indistinguishable from that ones of the chimpanzee isolates (Herchenröder *et al*, 1994). In addition, evidence of human foamy virus infection has been confirmed only in a few persons in contact with monkeys (Schweizer *et al*, 1995). Endogenous elements related to FV have been recently discovered (HERV-L; Cordonnier *et al*, 1995).

Foamy viruses are able to induce a persistent infection of the host but they are not conclusively associated with any disease. As mentioned in 1.6.2, FV have attracted recently the interest of many retrovirologists for some peculiarities which clearly differentiates the replication cycle of FV from any other retrovirus (Weiss, 1996b).

The morphology of this group is also characteristic, having long and evenly spaced surface projections (Gelderblom and Frank, 1987). The genomic organisation of FV is similar to that one of "complex" retroviruses with accessory genes such as *bel-1* which acts as a viral trans-activator (Löchelt and Flügel, 1995).

f) HTLV-BLV group

- prototype: bovine leukaemia virus

This group includes exogenous viruses such as bovine leukaemia virus (BLV) which is associated with B-cell lymphoma in cattle (Kettman *et al*, 1994) and human T-lymphotropic virus 1 (HTLV-I), associated with adult T-cell leukaemia and neurological disorders in man (Poiesz *et al*, 1980). Other components of this group are HTLV-II which has not been conclusively linked to any disease and simian T-

lymphotropic virus I (STLV-I) which is closely related to HTLV-I and infects non-human primates both in Africa and Asia (Fultz, 1994). No endogenous viruses belong to this group.

The morphology is similar type-C retroviruses in terms of virion appearance and assembly. Regulatory genes such as *tax* and *rex* in HTLV-I have been identified in this genus (Green and Chen, 1994). Of particular interest are the mechanisms of oncogenesis of HTLV-I and BLV which appear to differ from other oncoviruses (see 1.7.3)

g) Lentivirus group

- prototype: human immunodeficiency virus (HIV)

Lentiviruses are probably among the most extensively studied viruses of all time since HIV, the cause of the AIDS pandemic (Barre-Sinoussi *et al*, 1983; Gallo *et al*, 1984), is classified in this genus. Also included in this group are other viruses which induce immunodeficiency such as simian immunodeficiency virus (SIV) (Letvin *et al*, 1985) and feline immunodeficiency virus (FIV) (Pedersen *et al*, 1987). Lentiviruses are also associated with several other diseases such as neurological disorders in sheep and goats when infected with maedi-visna virus (MVV) (Narayan and Jolly, 1990) and caprine arthritis/encephalitis virus, CAEV (Crawford *et al*, 1980); arthritis induced by CAEV in goats; anaemia in horses infected with equine infectious anaemia virus (EIAV) (Montelaro *et al*, 1993) and others. No oncogenic viruses are included in this group.

Lentiviruses have a characteristic morphology with a bar or truncated cone-shaped core. Assembly occurs at the cell membrane as type-C retroviruses. The genomic organisation is that one of “complex” retroviruses with several regulatory genes.

No endogenous lentiviruses have been described.

1.6.4 Endogenous retroviruses.

A unique feature of retroviruses is their presence as inherited elements in the germline of most eukaryotes. These elements, known as endogenous retroviruses

(ERV), are transmitted through the germline as stable Mendelian genes, yet they exhibit structural and sequence similarities to infectious exogenous retroviruses (Stoye and Coffin, 1985).

The eukaryotic genome contains several sequences which have been generated by reversing RNA to DNA. This RNA-mediated movement of genetic material is called 'retrotransposition' and the transposed sequences are called 'retroelements' (Hull and Will, 1989). Retroelements can be classified into two groups according to the presence of the LTRs (Fig. 1.10). Retroelements without LTRs can be further divided into elements without reverse transcriptase, which may have used cellular mechanisms for reverse transcription, and elements with reverse transcriptase. The first category contains elements such as short interspersed elements - SINE (the *Alu* family for example) - and processed pseudogenes while the second category contains the long interspersed elements (LINEs). Both SINEs and LINEs have an internal promoter but only the LINEs have coding capacities possessing an open reading frame coding for an uncharacterised protein in addition to a *pol* gene (Hull and Will, 1989).

Retroelements with LTRs can be divided into 'retrotransposons' and 'retroviruses'. Retrotransposons have LTRs, *gag* and *pol* genes but lack the *env* gene and therefore can not be infectious. Retroviruses differ from retrotransposons by the presence of at least one additional gene, the envelope gene, which confers, after budding, the capacity to recognise a cellular receptor and start a new replication cycle. Thus, *env* adds to the retroelements the ability to spread between cells and individuals (Larsson, 1989; Wilkinson *et al*, 1994; Löwer *et al*, 1996; Leib-Mösch and Seifarth, 1996).

Many ERV have retroviral-like structures with LTRs, *gag*, *pol*, and *env*. It is assumed that ERVs derive from integration events during evolution of exogenous retroviruses into the germline of the host animal species. Another theory proposed by Temin (1992), envisages that ERVs have evolved from an RT gene predecessor through the acquisition of additional enzymatic activities as well as the successive association with promoters and sequences forming structural genes.

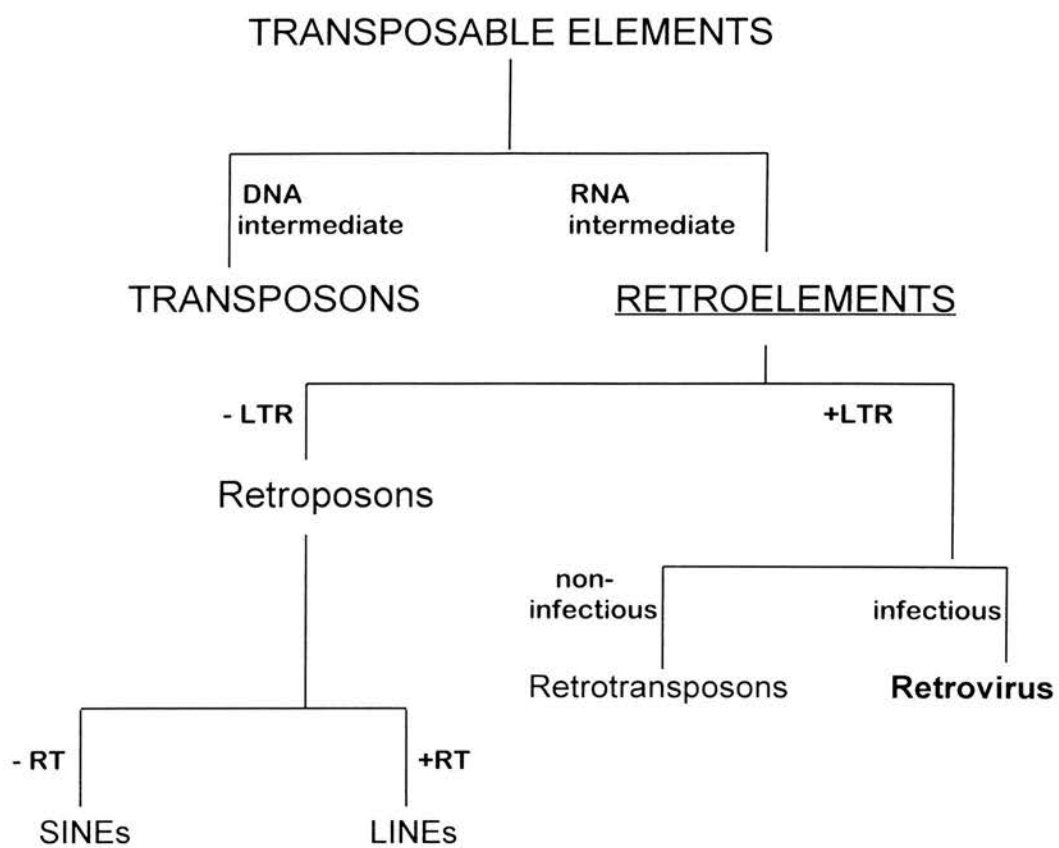


Fig. 1.10: **Classification of retroelements** (from Wilkinson *et al*, 1994).

The biological significance of these elements is debatable. The best definition is probably one that considers ERVs as a “type of selfish element specifically adapted to residence in the germline” (Coffin , 1990). However, both pathogenic and beneficial effects to the host have been envisaged for some ERVs.

Generally, endogenous proviruses are transcriptionally silent and are often defective, typically differing from the exogenous counterpart by deletions or point mutations that render them incapable to form infectious virus. In general ERVs are non-pathogenic; it is easy to understand that viruses of significant pathogenicity would be counterselected during the evolution. Exception to this rule are some endogenous MMTV such as *Mtv-1*, *Mtv-2* and *Mtv-4* [MMTV(SHN)] (Kozak *et al*, 1987) present in some strains of mice which have been selected for a high incidence of mammary tumours. *Mtv-1* is expressed as an infectious virus and is associated with late-occurring tumours (Van Nie and Verstraeten , 1975; Verstraeten and Van Nie, 1978) while *Mtv-2* is associated with tumours which occur early in life and are pregnancy dependent (Van Nie *et al*, 1977; Morris *et al*, 1986). *Mtv-4* is also associated with early tumour development (Imai *et al*, 1983; Luther *et al*, 1994).

A more complex example of ERV pathogenicity is that of spontaneous leukaemia in AKR mice caused by MCF viruses which are generated by recombination between endogenous murine retroviruses (Stoye *et al*, 1991; DiFronzo and Holland, 1993).

In humans, a particular interest is given to a class of ERVs called HERV-K10 (Ono *et al*, 1986) which contains open *gag*, *pol*, and *env* reading frames and possess a complex mRNA expression (Löwer *et al*, 1993; Mueller-Lantzsch *et al*, 1993). A high percentage of patients with seminoma have Gag- and Env-specific antibodies at high titre towards HERV-K10 while very rarely these antibodies are identifiable in healthy people or in patients with other tumours (Sauter *et al*, 1995 and 1996). Although no information is available regarding the role of HERV-K10 in the development of the tumour, these data indicate a possible role of HERVs in human pathology. The presence of HERV-K-related sequences packaged into retroviral particles in the placenta of Old World and New World monkeys (Simpson *et al*, 1996) makes this group of ERVs one of the more interesting to study.

As mentioned above, ERVs can also have a beneficial effect for the host. It has been shown that the expression of *env* proteins by endogenous viruses can block the receptors used by their exogenous counterparts therefore conferring protection to the host (Weiss, 1993). Chickens for example harbour several polymorphic *ev* loci and some of them express subgroup E envelope proteins which confer resistance to subgroup E RSV infection. This was first proposed by Payne *et al* (1971) who described a gene that was dominant for specific resistance to RSV subgroup E. A similar situation has been observed in some feral mice where the *Fv-4^r* locus blocks MLV-E receptors through an endogenous ecotropic gp70 synthesis (Kozak *et al*, 1984).

An interference mechanism through gag-like proteins, has recently been hypothesised after another of the MLV-resistant loci in mice, *Fv-1* (Lilly, 1970) was cloned and sequenced and found to be related to the *gag* region of HERV-L, an endogenous retrovirus unrelated to MLV (Best *et al*, 1996).

Another beneficial effect for the host has been suggested for the human endogenous retrovirus ERV-3 (Venables *et al*, 1995) which is a single-copy full-length HERV with an open reading frame for the Env protein. This open reading frame is conserved throughout primate evolution suggesting a beneficial effect to the host. In addition, ERV-3 is highly expressed in placental trophoblasts which is the cell layer separating mother and child (Boyd *et al*, 1993). Due to the similarity of the TM region of ERV-3 with a putative immunosuppressive region (termed p15E) (Haraguchi *et al*, 1995) of C-type retroviruses, it has been speculated that ERV-3 may serve to protect the foetus from immune attack by the mother (Venables *et al*, 1995).

Endogenous interference through the immune system has been well established in the MMTV model. In mice, endogenous MMTV expression of its *Sag* induces clonal deletions of most of the T cells expressing the respective reactive TCR V $_{\beta}$ (Kappler *et al*, 1988; MacDonald *et al*, 1988). Challenge of mice at birth with an exogenous MMTV expressing a homologous *Sag* will result in an abortive infection due to the lack of the *Sag* reactive T cells which in turn stimulate the amplification of the

infected B cells making in this way possible the colonisation of the mammary gland by MMTV. Thus endogenous MMTV can protect mice from infection by an exogenous MMTV expressing an homologous Sag sequence (Held *et al*, 1993; Golovkina *et al*, 1992).

A relevant biological characteristic of endogenous retroviruses is the possibility of recombination with their exogenous counterparts which often results in broadening the viral host range. This has been demonstrated for example in cats and mice with FeLV and MMTV respectively (Neil *et al*, 1991; Golovkina *et al*, 1994). Possible recombination between endogenous and exogenous viruses have to be taken into consideration when retroviral vectors are used for gene transfer.

Endogenous retroviruses thus deserve attention for both their potentially beneficial and harmful effects to the host and for the possibility of interacting with exogenous retroviruses.

1.7 Retroviruses and cancer.

Historically, retroviruses were first isolated and studied because they cause tumours in domestic animals. In 1911, Peyton Rous was able to experimentally reproduce a spontaneous chicken sarcoma by the inoculation of cell-free tumour homogenate. Since then, retroviruses have presented profound implications in the understanding of the multistep process which leads to oncogenesis of both viral or non-viral origin.

With regard to oncogenesis, retroviruses can be divided into two classes: 'acutely transforming retroviruses' and 'non-acute retroviruses' (Teich, 1984; Fan, 1994). The acute transforming retroviruses cause tumours rapidly (1 week to a few weeks) and are able to transform cells in culture, whereas the non acute retroviruses induce diseases slowly (months to years) and cannot transform target cells *in vitro*. In general, carcinogenesis has to be considered as a multi-step process and retroviruses can be responsible for one or more steps of the process. The different mechanisms of retroviral oncogenesis will be discussed in the following sections.

1.7.1 Acutely transforming retroviruses.

This class of retroviruses are probably among the most powerful carcinogens present in nature. They carry a cellular oncogene (known as proto-oncogenes or *c-onc*) into their genome which has been altered in its structure (deletions, point mutations etc.) and expression (Fig. 1.11) (Duesberg and Vogt, 1970; Stehelin *et al*, 1976; Bishop and Varmus, 1984; Rasheed, 1995). Proto-oncogenes are usually conserved over long evolutionary distances, suggesting that they are indispensable for some fundamental function that does not allow much changes (Benjamin and Vogt, 1990). The altered proto-oncogenes inserted into the viral genome are called 'viral-oncogenes' or *v-onc* and they always work through the synthesis of proteins (oncoproteins) which by definition have the potential to transform a normal cell into a neoplastic one.

Tumorigenesis by acutely transforming retroviruses therefore can be considered as the viral introduction of a powerful transforming gene into cells which induces them to be transformed into tumours. The discovery of the cellular origin of retroviral oncogenes is of paramount importance because it revealed that cellular genes could also play important roles in non-viral induced cancers.

Most oncogenes have homology with cellular genes which encode for proteins that in some way play a role in normal cell growth control and differentiation. For example, the *sis* oncogene of the simian sarcoma virus (SSV) encodes a protein which has 88% sequence identity with the β -chain of the human platelet-derived growth factor (PDGF) (Williams, 1986). Avian erythroblastosis virus (AEV) strain H carries *erbB* oncogene which has close homology to the chicken epidermal growth factor receptor (Downward *et al*, 1984). AEV strain ES4 carries, in addition to *erbB*, the *erbA* oncogene which is derived from a thyroid hormone receptor gene (Weinberger *et al*, 1986).

As mentioned above, structural changes of the proto-oncogene determine functional changes in the oncogene. However, for some oncogenes, such as *mos*, structural changes of the transduced proto-oncogene are not necessary for transformation as much as its over-expression (Wood *et al*, 1984).

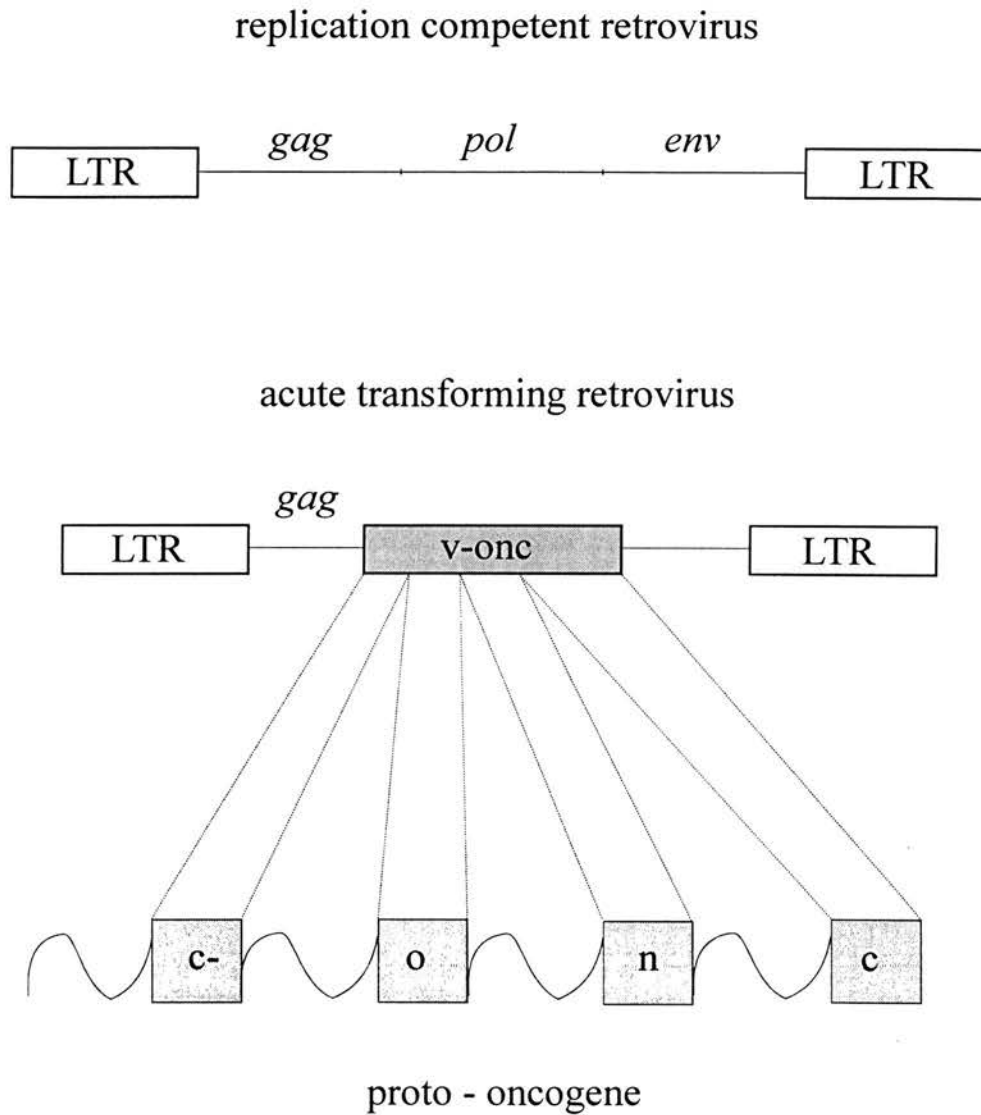


Fig.1.11: Genomic organisation of an acute transforming retrovirus compared to a replication competent retrovirus. Note the derivation of the viral oncogene (v-onc) from a proto-oncogene (c-onc). Often, viral oncogenes derive from exons of the proto-oncogene (modified from Fan, 1994).

As a result of the introduction of the proto-oncogene, the acutely transforming retroviruses lack portions of their structural genes and become replication-defective. Transforming retroviruses are thus propagated in conjunction with a replication-competent virus called “helper” because it synthesises all the proteins necessary to encapsidate the defective virus genome. An exception to this rule is given by RSV which contains its oncogene (*src*) at the end of the complete *gag-pol-env* and before the 3' LTR (Wang and Hanafusa, 1988).

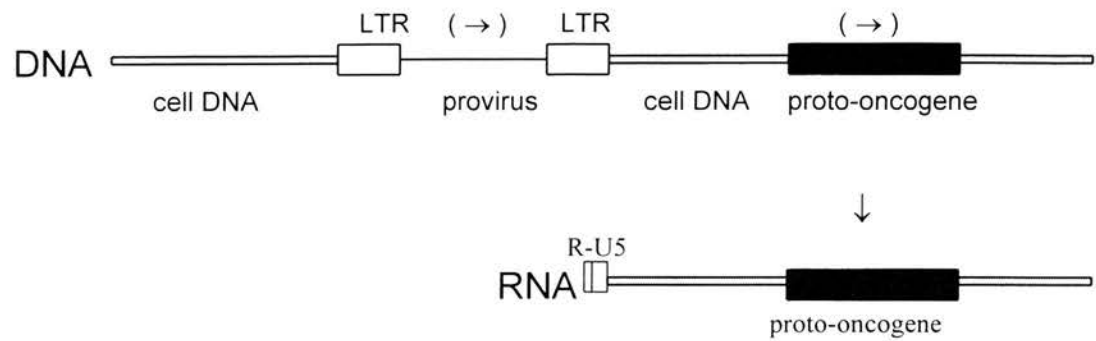
Although the introduction of a viral oncogene is probably one of the most powerful ways to transform a cell, usually it is not the only step required for tumorigenesis. For example, Abelson MuLV carries the *abl* oncogene but it is able to induce lymphoma only if the virus helper Moloney MuLV integrates in the proximity of a second cellular oncogene (*ahi-1*) (Poirier *et al*, 1988).

1.7.2 Oncogenesis by non-acute retroviruses: insertional activation.

Most retroviruses do not carry an oncogene in their genome but are still capable of inducing tumours in animals (Teich *et al*, 1984; Benjamin and Vogt, 1990; Kung *et al*, 1991; Fan, 1994). These viruses are called ‘non-acute retroviruses’ because the incubation period of the resulting neoplasia is usually of several months or even years. Non-acute retroviruses are replication-competent.

Tumour induction appears to occur through multiple steps with similarities to non-virus induced cancer. The best understood mechanism of nonacute retroviral carcinogenesis is LTR activation of a proto-oncogene also known as *cis*-activation or insertional activation or insertional mutagenesis. Most important in the genesis of this tumour is the fact that the provirus is integrated in the vicinity of a cellular proto-oncogene. Under the influence of the viral LTR, the transcription of the proto-oncogene is elevated and triggers the transformation of the normal cell. Two principal mechanisms of insertional activation have been identified: promoter insertion, and enhancer activation (Fig. 1.12).

Promoter Insertion



Enhancer Activation

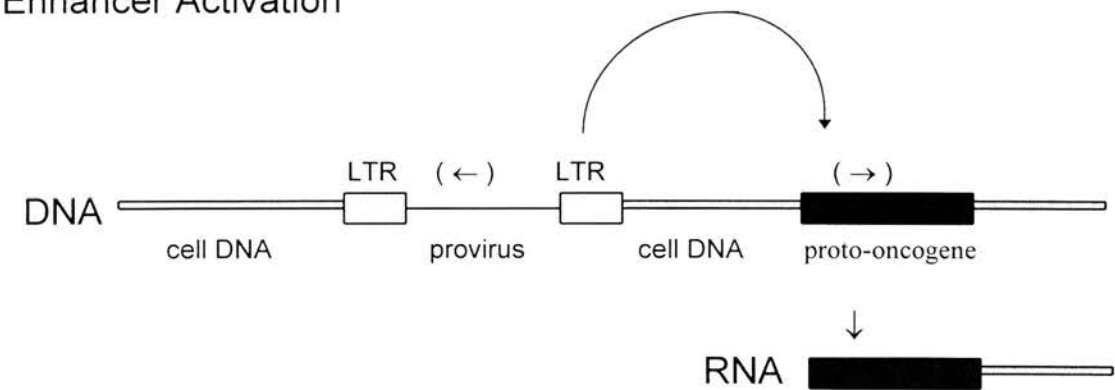


Fig. 1.12: **Insertional activation during non-acute retrovirus oncogenesis.** In the promoter insertion mechanism (top), transcription initiates in the proviral LTR and proceeds into the proto-oncogene coding sequences. In the enhancer activation (bottom), the LTR enhancer activates the protooncogene own promoter. In this case the provirus can also be in the opposite transcriptional orientation respect the proto-oncogene (modified from Fan, 1994).

In the promoter insertion mechanism, a chimaeric mRNA is formed by the viral LTR and sequences of the viral oncogene. The transcription can be driven by the 5' LTR (in this case viral sequences can also be present in the chimaeric transcript) or by the 3' LTR (in this case the provirus may have suffered extensive deletions which include the 5' LTR). In the enhancer activation mechanism, the enhancers present in the proviral LTR activate transcription of the proto-oncogene own promoter. In this case, the provirus can be inserted even several kb apart from the proto-oncogene and be in the opposite transcriptional orientation with respect to the proto-oncogene. This is consistent with the fact that enhancers are *cis*-acting DNA elements that can activate adjacent promoters independently from their position, orientation and distance.

Retroviruses which act by insertional activation generally induce tumours with a long incubation period because retroviral integration in the host DNA can be considered as a random event (Luciw and Leung, 1992). Thus, multiple rounds of infection are necessary for the virus to integrate near a proto-oncogene.

Several well studied retrovirus models act by insertional activation. ALV induces B-lymphomas by promoter insertion of the *c-myc* proto-oncogene (Hayward *et al*, 1981). ALV is also capable of inducing erythroblastosis in a certain line of chicken, by promoter insertion of *erbB* sequences (Fung *et al*, 1983).

Moloney MuLV activates *c-myb* in mice with myeloid tumours (Shen-Ono and Wolff, 1987; Nason-Burchenal and Wolff, 1993) but activates another set of proto-oncogenes in M-MuLV-induced T-lymphomas (*c-myc*; *pim-1*; *pim-2*; and *pvt-1*) (Cuypers *et al*, 1984; Selten *et al*, 1984; Steffen, 1984; Graham *et al*, 1985).

As a general rule, different viruses can induce the same kind of tumour by interacting with the same proto-oncogene. On the other hand, the same virus can induce different tumours by activating different proto-oncogenes. In addition, multiple proto-oncogenes can be activated in the same type of tumour by the same virus. MMTV integrates preferentially in proximity to a group of genes known as the *int* genes (*int-1/wnt-1*; *int-2*; *int-3*; *wnt-3*; *Fgf-4/Hst/k-FGF*) activated mainly by enhancer insertion (Nusse and Varmus, 1982; Dickson *et al*, 1984; Gallahan and Callahan, 1987; Peters

et al, 1989; Roelink *et al*, 1990). Some of the *int* genes have been shown to be important in normal development processes (Nusse, 1988; McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and normally they are not expressed in the mammary gland. The expression of these genes under the control of the MMTV LTR leads to an abnormal cell growth. It is interesting to note that most of the MMTV-induced tumours are hormone dependent in the initial stages of the disease. In the LTR of MMTV there are glucocorticoid response elements that bind the glucocorticoid receptor (Scheidereit and Beato, 1984) and MMTV transcription is strongly activated by glucocorticoids such as dexamethasone (Ringold *et al*, 1975).

Thus cancer has always to be considered as a multi-step event; in the MMTV system, hormones can therefore be considered a co-factor for tumorigenesis.

Besides the type of proto-oncogene activated, the pathogenicity and tumour specificity of nonacute retroviruses is strongly influenced by the virus LTRs and in particular by their enhancers (Fan, 1990; Athas *et al*, 1994). One of the best examples to explain this important concept is given by the MuLV system and by the generation of chimaeric viruses by molecular cloning. Moloney-MuLV causes T cell lymphoma while Friend MuLV causes erythroleukaemia. However, M-MuLV with F-MuLV LTRs will cause erythroleukaemia and viceversa (Chatis *et al*, 1983 and 1984). This is in line with the notion that the enhancers in the LTRs contribute to cell-type-specific transcription of retroviruses.

Another example of the presence in the LTR of specific pathogenic determinants is given by the MMTV system. As described above MMTV causes mammary tumours. However, it can also induce T lymphomas especially in males of some strains of mice that spontaneously activate endogenous MMTVs. MMTVs isolated from T lymphomas show deletions/alterations in the LTR which lead to high level expression in T lymphocytes and probably insertional activation of proto-oncogenes (although they have not yet been identified) (Michalides and Wagenaar, 1986; Lee *et al*, 1987; Hsu *et al* 1988).

Direct repeats of the enhancer elements seem to be a pathogenic determinant in nonacute retroviruses. One example is given by FeLV which causes a wide spectrum

of neoplastic and degenerative disease in domestic cats with the most common tumour being a thymic lymphosarcoma of T-cell origin (Rezanka *et al*, 1992). In a study conducted to compare the enhancer regions in different isolates it has been found that two or three copies of enhancer elements were present in all the thymic lymphosarcoma examined, in 38 to 66% of other lymphosarcomas, but only in 17% of animals with non-neoplastic disease. On the basis of these observations , it was suggested that enhancer repeats arise *de novo* during the infectious process and are associated with tumour induction (Matsumoto *et al*, 1992). The evolution of FeLVs with multiple enhancers could increase the probability of upregulating a host proto-oncogene during insertional mutagenesis.

An alternative way to induce tumour by non-acute retroviruses is the “inactivation” of cellular genes which normally regulate the cell growth. Friend virus-induced erythroleukaemia is a multi-step process. One of these steps is due to an abnormal expression or inactivation of p53, a cellular tumour suppressor gene (Ben-David and Bernstein, 1991; Marshall, 1991). Inactivation of p53 has also been observed in a proportion of non-B, non-T-cell lymphomas induced by Cas-Br-E virus (Bergeron *et al*, 1993). A similar mechanism of retroviral “inactivation” has been proposed in some tumours induced by MMTV where the expression of a cellular gene, denominated *int-6*, was found to be altered (Marchetti *et al*, 1995) by the integration of MMTV, although the normal function of *int-6* is still unknown.

1.7.3 Other mechanisms of retroviral oncogenesis.

Other mechanisms involved in retroviral carcinogenesis have been proposed for HTLV-I in adult T-cell leukaemia, and BLV in bovine leukaemia. Both viruses lack oncogenes and do not show common sites of viral integration in the induced tumours. The tumours are monoclonal or oligoclonal and viral expression in the tumour cells is minimal (Kinoshita *et al*, 1989). The tumours of both humans and cattle appear after a very long incubation period (many years) (Sugamura and Hinuma, 1993).

The HTLV model has been studied in great detail. HTLV contains at least two *trans*-regulatory genes, *tax* and *rex* (Green and Chen, 1994) which are essential for viral expression. *Tax* in particular seems to play a role in HTLV oncogenesis. *Tax* is a *trans*-activator of viral transcription and its function depends on *cis*-acting sequences, known as the Tax response element (TRE), located within the U3 region of the viral LTR (Felber *et al* 1985; Paskalis *et al*, 1986; Brady *et al* 1987; Rosen *et al*, 1987). Besides activating viral transcription, *tax* also activates the expression of cellular genes involved in T-cell growth and differentiation such as IL-2, IL-2 receptor, IL-3 and GM-CSF (Green and Chen, 1994). Many properties of *tax*, such as the ability to transform rodent fibroblasts (Tanaka *et al*, 1990) or to be oncogenic when expressed in mice as a transgene (Neremberg *et al*, 1987; Grossman *et al*, 1995), indicate the oncogenic potential of this gene and of its product. Analogies have been found with the Tax protein of BLV (Kettman *et al*, 1994).

However the precise mechanism by which HTLV transforms T lymphocytes is unclear mostly because *tax* is critical for viral replication and therefore it has been impossible to mutate *tax* in the context of an infectious virus and dissociate viral replication from cellular transformation. Recently, an infectious chimaeric HTLV-2 (HTLV_{c-enh}) that replicates by a *tax*-independent mechanism has been constructed by the replacement of the TRE with the cytomegalovirus immediate-early promoter (Ross *et al*, 1996). This chimaeric virus was able to maintain the capacity to transform human T lymphocytes *in vitro* with an efficiency similar to the wild type HTLV-2. However when the *tax* gene was deleted in a chimaeric clone (HTLV_{c-enh}ΔTax) the transformation properties were lost even though HTLV_{c-enh}ΔTax was still able to replicate. HTLV Tax protein has also been shown to interact with *int-6* which was found to be a common integration site for MMTV (Marchetti *et al*, 1995; Desbois *et al*, 1996). Taken all these data together it is possible to conclude that the viral *tax* plays a necessary functional role in T-cell transformation mediated by HTLV. However other genetic alterations are required within the transformed cell to maintain the phenotype considering that *in vivo* there is a very low HTLV expression (Ishibashi *et al*, 1987; Bhagavati *et al*, 1988; Kinoshita *et al*, 1989).

Presumably HTLV gene products are required to initiate transformation but not for maintaining the leukaemic phenotype.

Thus, the action of trans-activating proteins is another mechanism of retroviral tumour induction besides the most common insertional activation or transduction of oncogenes.

Structural proteins have also been implicated in retroviral tumorigenesis such as leukaemogenesis induced by the Friend erythroleukaemia complex virus. This virus mixture consists of an acute transforming virus (Friend spleen focus forming virus [F-SFFV]) and a helper virus (F-MuLV). The SFFV can induce erythroleukaemia in the absence of the helper virus by expression of an altered *gp55 env* protein which binds to the erythropoietin receptor rendering erythroid precursors independent of erythropoietin and stimulating autocrine growth (Li *et al*, 1990).

1.8 Jaagsiekte sheep retrovirus (JSRV).

The major candidate to be the aetiological agent of SPA is a chimaeric type D/B retrovirus known as jaagsiekte sheep retrovirus (JSRV) or sheep pulmonary adenomatosis retrovirus (SPARV) or ovine pulmonary carcinoma retrovirus (OPCRV). The term JSRV will be used throughout this thesis considering that this name has been used for the only complete nucleotide sequence so far published (see below, York *et al*, 1992) and in the recent literature (Bai *et al*, 1996; Hecht *et al*, 1996; Palmarini *et al*, 1995, 1996a and 1996b).

The first evidence for the association of a retrovirus with SPA derived from electron microscopy studies. The epithelial tumour cells of the lungs of sheep affected by SPA were shown to contain particles with a morphology consistent with the intracytoplasmatic type A particles (Perk *et al*, 1971; Bucciarelli, 1973; Sharp *et al*, 1983). In close proximity to the tumour cells, extracellular retroviral particles with a cylindrical core and short envelope projections (described either as type-B or -D) were also observed.

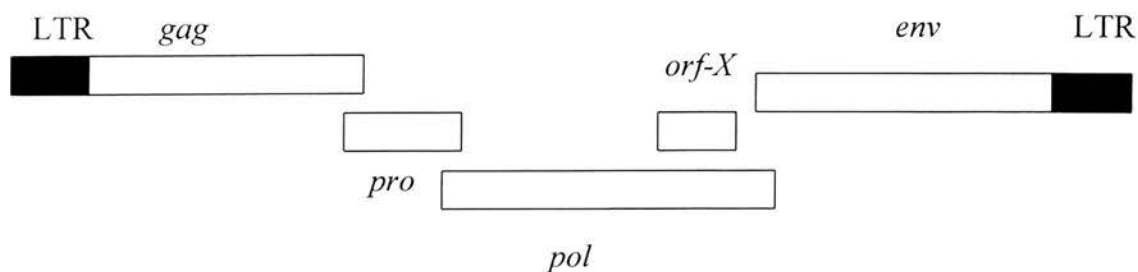
Reverse transcriptase (RT) activity was demonstrated in lung tumour and lung secretions of SPA affected sheep. RT activity was found to have preference for Mg^{2+} and to be associated with particles with a density in sucrose gradient similar to other type-B and -D retroviruses (Perk *et al*, 1974; Martin *et al*, 1976; Herring *et al*, 1983). SPA was reproduced experimentally in lambs by the inoculation of cell free tumour extract or lung fluids of SPA-affected sheep. The detection of RT activity was found to be present both in the inoculum used to reproduce SPA experimentally and in the resultant tumour lesions of the inoculated lambs (Martin *et al*, 1976; Verwoerd *et al*, 1985). In addition, the incubation period of the experimentally-induced SPA was reduced dramatically to a few weeks using new-born lambs inoculated intratracheally with ultracentrifuged and concentrated lung fluid collected from SPA-affected sheep (Verwoerd *et al*, 1980; Sharp *et al*, 1983); the incubation period was shown to be inversely related to the reverse transcriptase activity in the inoculum (Verwoerd *et al*, 1985).

A significant step towards the identification of the retrovirus associated with SPA was due to the detection in tumour homogenate and lung secretions of SPA-affected sheep of a 25 kDa polypeptide cross-reacting with antisera raised against the CA protein of MPMV and MMTV (Sharp and Herring, 1983). However, several attempts to isolate this virus in tissue culture failed (Sharp, 1987) hampering virological studies.

Finally, in 1992 the complete sequence of JSRV was obtained from a cDNA library constructed from isopycnic-gradient-purified virus derived from the lung secretions of a SPA-affected sheep (York *et al*, 1991 and 1992). New opportunities for research in this field were opened.

1.8.1 Genomic organisation of JSRV.

The genome of JSRV is 7,462 nucleotides long and exhibits a simple genetic organisation characteristic of the replication competent type D and type B retroviruses, with *pro* in a different open reading frame from *pol* (Fig. 1.13).



| Virus type | % Identical amino acids | | | |
|------------|-------------------------|------------|------------|------------|
| | <i>gag</i> | <i>pro</i> | <i>pol</i> | <i>env</i> |
| MPMV | 53.1 | 51.9 | 56 | |
| SRV-1 | 53.5 | 51.9 | 55.4 | |
| SMRV | 36.7 | 48 | 52.5 | |
| MMTV | 37.7 | 44.4 | 49.5 | 27.1 |
| HERV-K | 34.8 | 41.8 | 45.6 | 23.9 |

Fig. 1.13: **Genomic organisation of JSRV (top) and its similarity with other type D and B retroviruses (bottom).** Note the lack of similarity between the JSRV *env* gene and the *env* genes of other type D retroviruses (see page ix for abbreviations used).

Gag, *pro* and *pol* have around 50% (52 to 56 %) identity at the amino acid level with SRV-1 and MPMV and 38% (*gag*) to 50% (*pol*) identity with MMTV. JSRV Env proteins do not, in contrast, align reliably with the type-D retroviruses but are homologous to the env proteins of MMTV and HERV-K. This homology is not very high in terms of amino acid identities (24-27%), but the relationship is evident in terms of overall structural organisation, particularly for the second half of the molecules where numerous cysteines and glycosylation sites are shared (York *et al*, 1992).

In the JSRV sequence there is also present an additional open reading frame (*orf-x*) of 683 bp overlapping *pol*. The location and the codon usage of this open reading frame are very unusual, the predicted amino acid sequence is very hydrophobic and gives no meaningful homologies with sequences submitted to GenBank. It will be necessary to sequence different isolates of JSRV to establish if this open reading frame is a real viral gene or it has been generated by pure chance.

The LTRs of the provirus are 397 bases in length; U3 is 271bp long, R is 13bp and U5 is 113bp. In the U3, two 19 bp tandemly repeated sequences have been identified and a putative NF-1 binding site, and a core C/EBP (CAAT/ enhancer binding protein) putative binding site.

Phylogenetic analysis of the RT suggests that JSRV evolved from the A-B-D lineage and diverged from it before MPMV but after MMTV and HERV-K. In contrast, for the TM domain, JSRV groups with the type B clade (York *et al*, 1992). Therefore it is not straightforward to classify JSRV into the currently recognised seven genera of the family *Retroviridae*. Although the last ICTV report includes JSRV in the type D genus (Coffin *et al*, 1995), it is probably more correct to define JSRV a chimaeric type D/B retrovirus.

1.8.2 JSRV-related endogenous retroviruses of sheep.

York *et al* (1992), showed that the sheep genome contains endogenous JSRV-related sequences (*enJSRV*) by hybridisation with probes derived from JSRV cDNA clones.

Subsequent studies employing both *gag*, *pol* and *env* probes estimated the number of these sequences in the domestic sheep (*Ovis aries*) to be between 15 and 20 (Hecht *et al*, 1994). Several sheep breeds in different geographical locations such as Kenya, Peru and the United States were tested, indicating that probably all the sheep population contains these endogenous sequences as expected for an outbred animal species. Moreover, endogenous JSRV-related sequences were also found in goats (*Capra hircus*) (York *et al*, 1992), therefore these viruses became integrated into the germline before the speciation between sheep and goats. However, Hecht *et al* (1996) showed that the restriction pattern of *enJSRVs* in domestic and wild sheep was different from that one found in domestic and wild goats. This finding leads to the hypothesis that two distinct but closely related viruses became integrated into the germline of sheep and goats and much of the amplification of the original viruses in the respective genomes happened after the divergence of goats and sheep (4 to 10 million years ago) (Hecht *et al*, 1996).

The presence of *enJSRVs* does not permit straightforward interpretations about the detection of the exogenous JSRV in tumour and lung secretions of SPA sheep. JSRV could indeed derive from the reactivation of endogenous sequences prior to or after the neoplastic events and may not be aetiologically related to SPA. In support of this hypothesis, there are the results obtained by Hecht *et al* (1994) with Southern hybridisation analysis of SPA tumour and non tumour genomic DNA using a JSRV*gag* probe. No additional bands were detected in tumour DNA in comparison with non-tumour DNA. However the background formed by the endogenous copies could have masked additional sites of integration of the exogenous JSRV. In addition, that study did not employ tumour and non-tumour material collected from the same animal and therefore it does not allow conclusive interpretations due to possible minor variations among different individuals in the *enJSRV* restriction pattern.

EnJSRVs could play a role in the pathogenesis of SPA by influencing the immune response towards JSRV. Sheep with naturally or experimentally-induced SPA do not show detectable circulating antibodies (by western blotting) towards JSRV (Sharp *et*

al, 1983). This has led to speculation that endogenous JSRV antigens are expressed in the neonatal period during ontogeny, leading to the depletion of JSRV-specific lymphocytes and therefore establishing a state of tolerance for the endogenous and the exogenous virus (York *et al*, 1992).

1.9 Lentiviruses associated with SPA.

The earliest electron microscopy studies conducted on SPA tumour sections also showed retroviral particles with a type-C morphology (Perk *et al*, 1971). Subsequently, two groups in Israel and South Africa isolated a lentivirus from lung tumour of SPA-affected sheep (Irving *et al*, 1984; Payne *et al*, 1986) immunologically related to maedi-visna virus (MVV). The nucleotide sequence of one of these two isolates (South African - ovine maedi-visna virus [SA-OMVV]) confirmed that the virus was related to maedi-visna virus (Querat *et al*, 1990).

MVV causes a slow progressive fatal disease with pulmonary involvement (interstitial pneumonia) and/or neurological disorders known as maedi-visna (MV) (Narayan and Jolly, 1990), but it does not directly induce SPA. SA-OMVV failed to experimentally reproduce the tumour when inoculated intratracheally in sheep (Payne *et al*, 1986).

The isolation of a lentivirus in SPA tumours was not surprising considering that maedi-visna and SPA have been detected in the same flock and in the same sheep in several countries (Al-Zubaidy and Sokkar, 1979; Cutlip and Young, 1982; Deng *et al*, 1983; Snyder *et al*, 1983; Stevenson *et al*, 1982; Rosadio *et al*, 1988; Di Guardo *et al*, 1992) but in Britain, while SPA has been present throughout the century maedi-visna has been introduced only in the late seventies (Dawson *et al*, 1979).

SPA and MV have therefore to be considered as two separate entities. However, the close association of these diseases in different geographical locations could reveal interesting pathogenic mechanisms arising from a double retroviral infection. It has been proposed that the presence of an abundant population of macrophages in the SPA lesions could represent a large population of susceptible cells for MVV (Payne

et al, 1986; Dawson *et al*, 1990), considering that alveolar macrophages have been shown to be an important target for MVV (Narayan *et al*, 1982).

1.10 Enzootic nasal tumour virus of sheep and goats.

Enzootic nasal tumour (ENT) of sheep and goats is an infectious adenocarcinoma of the ethmoid turbinates (Cohrs, 1953) which shows several similarities to SPA. Like SPA, the tumour originates from secretory epithelial cells (De las Heras *et al*, 1991a) and it is experimentally reproducible by inoculation of cell free tumour homogenate or nasal secretions (in this case intra-nasal) (Cohrs 1953; De las Heras *et al*, 1995). Retrovirus-like particles have been observed by EM and RT activity has been detected in tumour extracts (McKinnon *et al*, 1982; De las Heras *et al*, 1991a; Vitellozzi *et al*, 1993; De las Heras *et al*, 1991b). Like JSRV, the retrovirus associated with nasal tumours of sheep and goats (ENTV) cross reacts with MPMV-CA indicating that ENTV has a type-D retroviral capsid (De las Heras *et al*, 1991b). Although no studies have been reported to determine if ENT and SPA are different manifestations of disease caused by the same agent, studies of ENT cases have not found any tumours in the lung, nor have nasal tumours been reported in SPA-affected animals (De las Heras *et al*, 1991; Vitellozzi *et al*, 1993).

1.11 Aims of the present project.

Research on the aetiology and pathogenesis of SPA has been hampered for years by the lack of an *in vitro* system for the propagation of JSRV and by the lack of appropriate immunological reagents and techniques for the detection of the virus. The availability of the complete sequence of one strain of JSRV opened new possibilities for research. However, the presence in the sheep genome of 15-20 copies of endogenous JSRV-related sequences has curtailed studies at the molecular level. The ultimate goal of the scientists working in the SPA field is to determine the pathogenesis and oncogenesis of SPA. In this thesis two basic questions were asked in order to help to understand what role JSRV plays in the aetio-pathogenesis of SPA:

- i) What are the sites of JSRV-replication in SPA-affected and unaffected control sheep?
- ii) What is the relationship between the exogenous JSRV and its endogenous counterparts?

Chapter 2.

General Materials and Methods

2. Introduction.

In this chapter, the general materials and methods which have been used throughout the work of this thesis will be described. To facilitate the reader, the following chapters (3 to 6) will also contain a 'Materials and Methods' section where methods unique to that single chapter will be described.

2.1 Animals employed in this study.

In this study, SPA sheep were defined as animals which showed typical clinical signs, particularly the production of an abundant sero-mucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head. Diagnosis was confirmed by macroscopic and histological examination of the lungs.

The histological diagnosis was conducted either at the Histopathology Diagnostic Service at Moredun Research Institute or by Dr. M. De las Heras (Zaragoza University, Spain).

Sheep with naturally acquired SPA were obtained from farms throughout Scotland and Northern England. Frozen tissues collected from SPA-affected sheep in Spain were obtained from Dr. M. De las Heras. Frozen tissues from SPA-affected sheep and goats with enzootic nasal tumour were obtained from Prof. G. Vitellozzi (University of Perugia, Italy) and Dr. M. De las Heras.

Unaffected control animals were obtained from the Moredun Research Institute's farm; in these animals SPA was ruled out by the absence of the characteristic macroscopical and histological lesions in the lungs.

The number of animals and the tissues employed in each experiment will be specified in the relevant chapters.

2.1.2 Experimental reproduction of SPA.

Eight one-day old lambs were experimentally infected in order to increase the availability of infected tissues for this thesis and for related projects.

Lambs were infected as described previously (Sharp *et al*, 1983). Briefly, lung fluid collected from several SPA-affected sheep was pooled, filtered through a double layer of sterile gauze and clarified by centrifugation at 10000 x g for 1 h at 4 °C. The supernatant was ultracentrifuged at 100000 x g for 1 h at 4°C. The resultant pellet was resuspended in a volume of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄-7H₂O, 1.4 mM KH₂PO₄, pH 7.3) equivalent to 1/15 that of the initial volume. Lambs were inoculated intratracheally with 5 ml of this suspension. Five age and breed matched controls were inoculated with 5 ml of PBS.

Lambs were killed after 4-16 weeks. All the eight infected animals developed SPA. Sterile instruments were used to dissect each individual organ at post-mortem to avoid cross-contamination. Tissue samples were snap-frozen in liquid nitrogen and stored at -70°C.

Tissues collected from lambs experimentally infected in the past years at MRI and stored at -70°C were also employed in this study.

2.2 Bacteriological techniques.

Strains DH1 (Hanahan, 1984) and JM109 (Yannisch-Perron *et al*, 1985) of *E. coli* were used in this thesis for cloning purposes. Bacteria were grown in L-broth (10g tryptone, 5g yeast extract, 5g NaCl for litre of solution) or L-agar (L-broth containing 15g agar per litre).

Ampicillin was used at a concentration of 50 µg per ml when required.

2.2.1 Preparation of competent *E. coli*.

Competent cells were either purchased (JM109 High Efficiency Competent Cells; Promega) or prepared by the calcium chloride method (Sambrook *et al*, 1989). Briefly, 10 ml of L broth was inoculated with a single colony of DH1 and incubated at 37°C in a shaking incubator overnight. A 500 µl aliquot of overnight culture was added to 50 ml of L-broth and incubated at 37°C until the OD₆₀₀ of the culture reached 0.6. The culture was centrifuged at 2000 x g for 20 min at 4°C and the bacterial pellet resuspended in 25 ml ice-cold 100 mM CaCl₂ and incubated on ice for 20 min. Bacteria were pelleted by centrifugation as above, and resuspended in 5 ml 100 mM CaCl₂. After 10 min on ice the bacteria were ready for transformation. Alternatively, after the addition of 15% glycerol, cells were snap frozen in liquid nitrogen and stored at - 70°C.

2.3 Molecular biology techniques.

2.3.1 Optimisation of PCR reactions and precautions taken to avoid cross-contamination.

Each PCR reaction described in this thesis was optimised by varying several parameters of the reaction like MgCl₂ concentration and pH of the reaction buffer, primer and dNTP concentrations, annealing and extension temperature and number of cycles (Innis and Gelfand, 1990; Kidd and Ruano, 1995).

The computer program 'Primer' of the GCG package was used as an aid to select the correct combinations of primers (Genetics Computer Group, 1994).

Contamination of PCR was checked by the use of appropriate distilled water controls in each step of the work. Isolation of nucleic acids, preparations of PCR reactions and analysis of PCR products were conducted in separate rooms. Sterile instruments were used to dissect each individual organ at post-mortem to avoid cross-

contamination between different tissues. Samples were placed in individually sealed plastic bags prior to being snap-frozen and stored at -70°C.

2.3.2 Agar gel electrophoresis of DNA.

DNA (plasmid preparations, PCR products, genomic DNA) was analysed by conventional agarose gel electrophoresis. Agarose concentrations ranged from 0.8 to 2.0% w/v in TBE electrophoresis buffer (45 mM Tris/borate, 1 mM EDTA pH 7.3) in the presence of 0.5 µg of ethidium bromide per ml. Gels were run in TBE buffer at 30-100 V. DNA fragments were visualised by their fluorescence under UV illumination. The size of the DNA fragments was determined by comparison with known commercially available molecular weight standards (DNA molecular weight standard VI, VII and IX [Boehringer]).

A record was obtained by photographing the gels on heat sensitive paper using a video camera (Mitsubishi video copy processor, UVP).

2.3.3 Purification of DNA fragments from agarose gels.

PCR products utilised for cloning were electrophoresed in a low melting point agarose (Boehringer), identified by UV illumination, purified by excision of the band of the expected size and subsequent standard phenol/chloroform extraction.

Briefly, after excision the agarose band was incubated at 67 °C for 15-30 min until the agarose slice was completely melted. Five volumes of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) were then added, followed by the addition of an equal volume of water saturated phenol. Samples were mixed and centrifuged at 13,500 x g for 5 min. The aqueous phase was collected, put in fresh microfuge tubes and an equal volume of phenol was added for a second time and centrifuged as above. The aqueous phase was removed and to this was added one volume of phenol/chloroform/isoamyl-alcohol (25:24:1) and centrifuged again. The last extraction was then performed adding chloroform/isoamylalcohol (24:1) to the

collected aqueous form. After another centrifugation the aqueous phase was collected and 0.1 volumes of 3 M sodium acetate pH 4.8 and two volumes of ethanol added. DNA was then precipitated at -20°C for at least one hour and subsequently centrifuged at 13,500 x g for 20 min at 4 °C. Pellets were washed twice with 70% ethanol, dried and resuspended in distilled water.

Alternatively, due to the high purity of the low melting point agarose, a variation of the “freeze and squeeze” method (Polamn and Larkin, 1989) was also used employing Costar’s Spin-X tubes™. These tubes are formed by an upper and a lower chamber separated by a microfilter. Excised agarose slice were put into the upper chamber and incubated at -20°C for 15 min. Afterwards the tubes were spun at 5,600 x g for 30 min. During the centrifugation, the agarose is retained in the upper chamber while the DNA (in TBE) passes through the filter in the lower chamber ready to use.

2.3.4 Quantification of DNA and RNA.

Nucleic acid was quantified by the use of a spectrophotometer. DNA and RNA have a peak of absorbance at 260 nm, while contaminating proteins have their peak at 280 nm (Sambrook *et al*, 1989). The absorbance at 260 nm reflects the amount of nucleic acid present in the sample. An absorbance of 1 OD₂₆₀ corresponds to 50 µg ml⁻¹ of double stranded DNA and 40 µg ml⁻¹ for RNA. The ratio OD₂₆₀/OD₂₈₀ should be between 1.8 and 2.0 for a clean nucleic acid preparations. Samples were diluted to give an OD₂₆₀ between 0.1 and 0.5 to be within the range of accuracy of the spectrophotometer. An automatic spectrophotometer (Beckman DV650) was used in this study. This has a software which automatically calculates the concentration of nucleic acid and the OD₂₆₀/OD₂₈₀ ratio.

In some cases, when the amount of DNA available was very limited, the estimation of DNA was carried out by visual comparison of the fluorescence of electrophoresed DNA samples with known weight DNA standards in an agarose gel electrophoresis.

2.3.5 Southern blotting.

Southern blotting analysis (Southern, 1975) was performed on PCR products to confirm their specificity by hybridisation with oligonucleotide probes internal to the PCR products. The DNA was denatured by soaking the agarose gel in 0.4 M NaOH for 5-10 min. The DNA samples were then transferred in a 0.4 M NaOH buffer (Reed and Mann, 1985; Ausbel *et al*, 1992) by capillary action overnight onto nylon filters (Hybond-N, Amersham International). Filters were then rinsed briefly in 2 x SSC (20x SSC = 3M NaCl, 0.3M sodium citrate, pH 7) to remove any possible piece of agarose and the DNA fixed either by baking at 80°C for 2 hours or by UV crosslinking using a Stratalink UV transilluminator (Stratagene). Filters were then used straight away for hybridisation or wrapped in a plastic bag and kept in the dark at room temperature for further use.

2.3.6 Labelling of oligonucleotide probes.

Oligonucleotides used for hybridisation were labelled with either ^{32}P or digoxigenin (DIG).

Synthetic oligonucleotides were labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using T4 polynucleotide kinase which catalyses the transfer of the γ -phosphate group from ATP to the 5'-OH termini of the oligonucleotide. Reactions were carried out in a total volume of 20 μl composed of 1 μl of oligonucleotide (10 μM), 1 μl polynucleotide kinase (MBI Fermentas), 4 μl 5 x reaction buffer (250mM Tris-HCl pH 7.6, 50 mM MgCl_2 , 25 mM DTT, 0.5 mM spermidine, 0.5 mM EDTA), 5 μl $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity 5000 Ci/mmol; 10 mCi/ml in aqueous solution), 9 μl distilled water. Reactions were incubated at 37°C for an hour. The efficiency of ^{32}P transfer was determined by chromatography by spotting 0.5 μl of labelling reactions into the lower end of a strip of cellulose impregnated with polyethyleneimine (Polygram® CEL 300PEI). The bottom of the strip was then dipped into 750 mM solution of K_2PO_4 , pH 3.5. After the buffer had migrated about 10-20 cm, the strip was wrapped in Saran Wrap and

autoradiographed. The film was developed after about 30 min. ^{32}P labelled oligonucleotides are visualised as a spot remaining at the origin while unincorporated ^{32}P -ATP migrates up the strip and it is seen as a smear.

Accurate calculations can be done by measuring the amount of radioactivity at the origin and on the total strip. This has not been necessary for the hybridisation purposes during the course of this thesis.

The unincorporated ^{32}P was removed from the radiolabelled oligonucleotides using a Sephadex® G-50 DNA grade Nick Column (Pharmacia) according to the manufacturer's protocol.

DIG-labelled (5'-DIG) oligonucleotides were purchased from Oswel DNA Service.

2.3.7 Hybridisation.

With ^{32}P oligonucleotide probes, hybridisation was performed overnight in a hybridisation buffer composed of 4 x SSC, 0.1% sodium pyrophosphate, 0.2% SDS, 50 $\mu\text{g}/\text{ml}$ heparin (Singh and Jones, 1984). Alternatively, 'rapid hyb' buffer (Amersham) was also used.

Hybridisation using DIG-labelled oligonucleotides was performed using 'DIG easy Hyb' buffer (Boehringer).

Filters were hybridised in tubes in a Hybaid oven with a rotating wheel. Temperature of hybridisation varied depending on the melting temperature of the oligonucleotide used. Filters were washed at high stringency as follow: twice with 2 x SSC (0.1% SDS) at room temperature for two minutes, three times with 1 X SSC (0.1% SDS) at the hybridisation temperature for 15 min each, followed by the three washes with 0.1% SSC (0.1% SDS) for 15-20 min each at the hybridisation temperature. For the washings, the filters were put in a tray and excess washing buffer was used. This is particularly true for filters hybridised with DIG-labelled probes where the use of 400-500 ml of washing buffer for each washing decreased the non-specific background.

2.3.8 Autoradiography.

Detection of ^{32}P labelled probes was done by autoradiography. After the last wash the filters were wrapped in Saranwrap™ and autoradiographed at -70°C using Kodak X-Omat S films.

2.3.9 Detection of DIG-labelled probes.

Immunochemical detection of DIG-labelled oligonucleotides bound to an immobilised target was performed using the 'DIG nucleic acid detection kit' (Boehringer) essentially as described by the manufacturers. Briefly, filters were washed in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (buffer 1) plus 0.3%, Tween 20. Non-specific binding was blocked by incubating the membrane for 30 min in buffer 1 containing 1% blocking reagent (buffer 2). Filters were then incubated for an hour at room temperature with an anti-DIG alkaline phosphatase conjugate (1:5000 v/v in buffer 2). Unbound conjugate was removed by two washes (thirty minute each) with an excess of buffer one. Filters were then equilibrated in 100 mM Tris/HCl, 150 mM NaCl, 50 mM MgCl_2 , pH 9.5. The enzyme substrates were added; 45 μl NTB (75 mg/ml nitroblue tetrazolium salt in dimethylformamide) and 35 μl X-phosphate (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in dimethylformamide). Filters were put in a box in the dark until the colour developed (2-16 hours).

2.3.10 pGEM-T cloning vector.

In this thesis recombinant DNA techniques have been used solely to clone PCR products. A convenient vector to facilitate PCR cloning is represented by pGEM-T (Promega). The pGEM-T vector is prepared by the manufacturer cutting pGEM-5Zf (Promega) with *EcoRV* and adding a 3' terminal thymidine to both ends. The overhang 3'-T at the insertion site improve the efficiency of ligation of a PCR

product into the plasmid because of the non-template dependent addition of a single deoxyadenosine to the 3'-end of PCR products by *Taq* DNA polymerases (Clark, 1988). pGEM-T contains the multiple cloning site in the α -peptide coding region of the enzyme β -galactosidase. When the appropriate strains of *E. coli* are used, insertional inactivation of the α -peptide allows recombinant clones to be directly identified by colour screening on plates containing X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; a lactose analogue) and IPTG (isopropyl-thiogalactoside; an inducer of the *lac* operon). The non-recombinant colonies will synthesise β -galactosidase, which breaks down X-gal to a product that is coloured deep blue (blue colonies), whereas the recombinant colonies will be unable to produce β -galactosidase and will be white.

2.3.11 Ligation of the pGEM-T vector and PCR product.

Ligation reactions were performed using the enzyme T4 DNA ligase which catalyses the formation of a phosphodiester bond between a 5' phosphate group and a 3' hydroxyl group resulting in the covalent linking of two DNA molecules which have compatible ends. Ligation reactions were set up in a total volume of 10 μ l ligation buffer (30mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 10 mM DTT; 10 mM ATP) containing 50 ng of vector, insert DNA at a insert:vector ratio of approximately 3:1 and 1 μ l T4 DNA Ligase (Promega). Parallel controls were performed using a control insert supplied by Promega. Ligation reactions were incubated at 15°C overnight.

2.3.12 Transformation of competent *E. Coli*.

A 100 μ l aliquot of competent bacterial cells (prepared as described in 2.2.1) were mixed with 5 μ l of the ligation reaction and placed on ice for 20 min. The mixture was then heated for 45-50 seconds at 42°C. A 500 μ l aliquot of L-broth plus 10 mM $CaCl_2$ was then added and the cells were incubated at 37 °C for one hour. At the end of the incubation period, 200 and 100 μ l of each sample was spread on L-agar plates

containing ampicillin (100 µg/ml), X-gal (80 µg/ml) and IPTG (0.5 mM). The plates were incubated at 37°C overnight. White colonies were picked with a sterile toothpick, inoculated into 10 ml of L-broth containing ampicillin and incubated in a shaking incubator at 37°C overnight.

2.3.13 Small scale preparation of plasmid DNA.

Small scale preparation of plasmid DNA was performed essentially as already described (Serghini *et al*, 1989).

Ten ml of overnight bacterial culture was centrifuged at 3,800 x g for 20 min at room temperature. The bacterial pellet was resuspended in 700 µl distilled water and transferred to a microcentrifuge tube. One volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, the samples were thoroughly mixed by vortexing and centrifuged at 13,500 x g for 5 min. The aqueous layer was transferred to a clean tube and 60 µl of 3M sodium acetate (pH 4.8) and 1 ml of ethanol was added. After incubation at -20°C for at least one hour, DNA was precipitated by centrifugation at 13,500 x g for 20 min at 4°C. The resultant pellet was washed twice with 70% ethanol. The final pellet was air dried, resuspended in 20 µl of 10mM Tris-HCl, pH 7.5, 1 mM EDTA plus 5 µl RNase A (20 mg/ml, Sigma) and incubated at 37°C for half an hour.

Alternatively, the QIAprep™ kit (Quiagen) was used according to the manufacturer's protocol. The QIAprep plasmid kit procedure is based on a modified alkaline lysis (Birnboim and Doly, 1979) where the bacterial cells are lysed in NaOH/SDS buffer in an optimised lysis time which allows maximum release of plasmid DNA without release of chromosomal DNA. After neutralisation the plasmid DNA is adsorbed onto silica gel in the presence of high salt (Vogelstein and Gillespie, 1979) and after washings the DNA is eluted by TE buffer or distilled water.

2.3.14 Restriction endonuclease digestion of DNA.

Restriction enzymes were purchased from Boehringer and used with the appropriate incubation buffers as recommended by the manufacturer. The final concentration of the enzymes was at least 10 units per μg of DNA.

2.3.15 Sequencing.

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al* (1977) using the Pharmacia ^{T7} Sequencing kit™ according to the manufacturer's protocol.

Double stranded plasmid DNA was denatured in alkaline solution prior to the annealing reaction. Approximately 2 μg of plasmid DNA in a volume of 22 μl was incubated at 37°C for an hour with an equal volume of 0.4 M NaOH, 4 mM EDTA. The mixture was then neutralised by the addition of 4.4 μl sodium acetate and 100 μl of ethanol and precipitated at -20°C for at least one hour. DNA was pelleted by centrifugation, washed twice with 70% ethanol and air dried. DNA was finally dissolved in 10 μl distilled water ready for annealing.

Two set of sequencing reactions were performed respectively with a 'forward' and a 'reverse' primer to allow sequencing of both DNA strands. Initially, 10 μl of template were added to 2 μl of the forward or the reverse primer (5 pmol/ μl) and 2 μl of annealing buffer. This mixture was incubated at 65 °C for five minutes, 37°C for 10 min and 5 min at room temperature. The following reagents were added to the primed template: 3 μl of labelling mix (1.375 μM each of dCTP, dGTP, dTTP in 333 mM NaCl), 1 μl of ³⁵S-dATP (10 μCi) and 2 μl of T7 DNA polymerase diluted 1:5 in enzyme dilution buffer which contains glycerol, bovine serum albumin and DTT in Tris/HCl, pH 7.5. Following incubation at room temperature for five minutes, 4.5 μl aliquots were added to each of four tubes (a, g, c, t) which contained 2.5 μl of the corresponding dideoxynucleotides and non-limiting concentrations of all four deoxynucleotides. After five minutes incubation at 37°C, the reactions were stopped

by the addition of 5 µl of stop solution (deionised formamide containing 10 mM EDTA, xylene cyanol and bromophenol blue dyes) and the reactions were kept on ice until loading.

2.3.16 Sequencing gel electrophoresis.

Sequencing gel electrophoresis was carried out in an IBI model STS 45 sequencing tank. A 6% polyacrylamide gel was prepared by dissolving 42g of ultrapure urea in 20 ml of 30% w/v acrylamide / 0.8% w/v bis-acrylamide stock solution, 10 ml of 10xTBE buffer and water to 100 ml. Polymerisation was started by the addition of 150 µl of 25% w/v ammonium persulphate and 150 µl of N,N,N,N-tetramethylethylenediamine (TEMED). The running buffer was 1 x TBE and the gel was pre-run for 30-40 min at 80W to heat to 50°C. The samples were denatured at 94°C before loading. A 2.5 µl aliquot of each sample was loaded. After electrophoresis for 2-6 h the gel was fixed in 10 % v/v methanol, transferred to a supporting sheet of Whatman® 3MM filter paper, covered with Saranwrap™ and dried under vacuum at 80 °C. The dry gel was exposed to Fuji Medics X-ray film for 12 to 36 hours.

2.3.17 Sequence analysis.

Sequence analysis was done using various softwares of the Genetics Computer Group package (Deveroux *et al*, 1984; Genetics Computer Group, 1994) on the SEQNET facility of the Science and Engineering Research Council, Daresbury Laboratory, Warrington, England.

2.3.18 Preparation of total cellular RNA.

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method as already described (Chomczynski and Sacchi, 1987).

Briefly, solid tissues (0.2 - 0.5 g) were pulverised with a pestle and mortar under liquid nitrogen and each tissue was added to 500 µl of solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M β-mercaptoethanol) in a 1.5 ml microfuge tube. Ultraspeed pellets of lung fluid (2.5 to 5 ml) or 10⁷ cells (leukocytes or other cells) were suspended directly in 500 µl of solution D. The pH of the solution was then lowered by the addition of 50 µl of sodium acetate, pH 4. Five hundred µl of water-saturated phenol was then added followed by the addition of 100 µl of chloroform/isoamyl alcohol (49:1). The samples were then placed on ice for 15 min. Between each step the samples were mixed by vortexing. Samples were then centrifuged for 20 min at 13000 x g in a refrigerated microfuge at 4 °C. The upper aqueous layer was transferred to a new microfuge tube and an equal volume of isopropanol was added. The samples were then placed at -20°C for at least 2 hours. In lung fluid samples; 20 µg of glycogen (Boehringer) or 2 µg of MS-2 RNA (Boehringer) was added as a carrier.

The RNA was pelleted by centrifugation at 17000 g for 20 min. The RNA pellets were washed twice with 75% ethanol and then dried. RNA pellets were resuspended in 20-50 µl of distilled water.

To avoid RNase contamination, distilled water and solutions used for RNA extraction and analysis were treated with 0.1% of diethylpyrocarbonate (DEPC) overnight followed by autoclaving to inactivate the DEPC.

Each RNA sample was treated with 10 U of DNase I, RNase free (Boehringer) for 30 min at 37 °C in a solution containing 50 mM sodium acetate , pH 6.5, 10 mM magnesium chloride and 80 U of RNasin (Promega). DNase was inactivated by 94°C for 3 min or the RNA was re-extracted by the phenol-chloroform method.

2.3.19 PolyA⁺ RNA selection.

Isolation of polyA⁺ RNA was performed employing the Micro-FastTrack™ kit (Invitrogen) as recommended by the manufacturer. Briefly, tissues were pulverised with a pestle and mortar under liquid nitrogen and 200 mg were added to a lysis

buffer containing RNase/protein degrader in a microfuge tube. After incubation at 45°C for a time variable between 20 min and 2 h the lysate was applied directly to oligo (dT) cellulose for adsorption. The DNA, degraded proteins and cell debris were washed off with a high salt buffer. Non polyadenylated RNA was washed off with a low salt buffer and the polyA⁺ RNA then eluted in the absence of salt.

2.3.20 RNA electrophoresis.

The RNA concentration of the samples was estimated spectrophotometrically at 260/280 nm using a Beckman DV650 spectrophotometer.

The quality of the RNA extracted was assessed by running an aliquot of the sample (2 µl) in a 1% denaturing formaldehyde/agarose gel (Fig. 2.1[a] and [b]). The gels were prepared by dissolving 0.4g of agarose in 34 ml of distilled water and heating in a microwave oven. Once the agarose had cooled to approximately 65°C, 4 ml of 10x MOPS buffer (0.2M MOPS 3-[N-Morpholino] propanesulphonic acid (Sigma), 50 mM sodium acetate and 10 mM EDTA pH7) and 2 ml of formaldehyde (39%, Sigma) were added. Electrophoresis was performed in a horizontal gel apparatus using 1x MOPS as running buffer. A 2 µl aliquot of the RNA samples were added to 6 µl of sampling loading buffer (750 µl formamide, 150 µl 10x MOPS, 240 µl formaldehyde, 100 µl glycerol, 0.10 µl 10% (w/v) bromophenol blue, 10 µl ethidium bromide [10 mg/ml], 100 µl distilled water) and denatured at 65 °C for 10 min prior to loading on the gel. Electrophoresis was carried out at 80 V for approximately 30 min. RNA was then visualised with a UV transilluminator.

The presence of two sharp bands representing the 18S and 28S ribosomal RNA is an index of a good quality preparation. An example of the quality of the total cellular and the poly A⁺ selected RNA preparations obtained is given in figure 2.1.

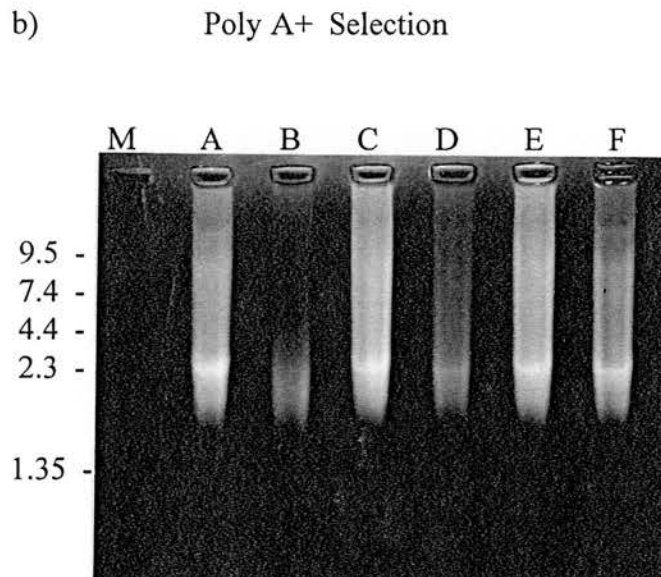
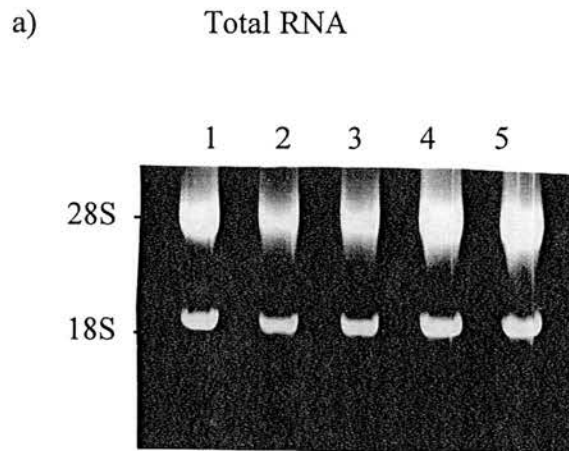


Fig. 2.1: Agarose gel electrophoresis of RNA. (a) shows an example of the total cellular RNA preparations obtained during this study; about 95% of the total RNA is represented by the ribosomal RNA which is visible in the figure as the 28S and the 18S subunits. Lane 1, lung tumour; lane 2, spleen; lane 3, kidney; lane 4, mediastinal lymph node; lane 5, thymus. Poly A⁺ selected RNA is shown in (b) as a smear. Lane A, B and C, mediastinal lymph node; lane D, kidney; lane E, spleen; lane F, lung. Lane M, Gibco RNA molecular marker (numbers represent kilobases).

2.3.21 Preparation of genomic DNA.

High molecular weight DNA from sheep tissues was obtained employing the QIAamp Tissue Kit® (Quiagen). This kit was chosen because it allows the handling of several samples at the same time. Solid tissues were ground under liquid nitrogen using a pestle and mortar. The tissues (10-25 mg) were then added to 180 µl lysis buffer (Quiagen buffer ATL) and 20 µl of proteinase K (20 mg/ml), mixed by vortexing and incubated at 55°C overnight. A 20 µl aliquot of RNase A (20 mg/ml) was then added and the samples were incubated at room temperature for two minutes. Buffer AL was then added and the samples were incubated at 70 °C after mixing by vortexing. After the addition of 210 µl of ethanol the samples were placed in QIAamp spin column which contains a silica gel membrane. Salt and pH conditions given by the different buffers used ensure the DNA is adsorbed onto the silica membrane while protein and other contaminants flow through after centrifugation at 6000 x g for one minute. After a series of washing with buffer AW the samples were eluted with pre-heated (70 °C) buffer AE.

Some of the tumour and kidney DNA samples collected from SPA sheep were obtained from Dr. C. Cousens (MRI, Edinburgh) and were prepared by a standard proteinase K-phenol extraction (Sambrook *et al*, 1989).

Chapter 3.

Localisation of JSRV Sites of Replication by the Development of Immunological Techniques.

3.1 Introduction.

Although the association between JSRV and SPA has been established for a number of years (see 1.8), the role of this virus in the pathogenesis of SPA is not understood. The sites of viral replication are largely unknown and some indications have been obtained only by ultrastructural observations of retrovirus-like particles in the tumour cells, or in close association with them, in the lungs of SPA-affected animals (Perk *et al*, 1971, Bucciarelli, 1973; Sharp *et al*, 1983). The principal problem in identifying the sites of JSRV replication has been the lack of appropriate reagents and techniques for the detection of the virus.

Western blotting, employing a goat antiserum towards the CA protein of MPMV, has been the main technique used successfully for the detection of JSRV (Sharp and Herring, 1983; Herring *et al*, 1983). With this technique it has been possible to detect JSRV in lung fluid and in most of the lung tumour extracts of SPA-affected sheep (Sharp and Herring, 1983; Herring *et al*, 1983; Rosadio *et al*, 1988a and 1988b; J.M Sharp, personal communication). The sensitivity of this test is decreased by the use of a heterologous antiserum and in practice it has been necessary to concentrate the infected samples (lung tumour, lung fluid) several fold to obtain a clear result. A heterologous competition radioimmunoassay employing ¹²⁵I-labelled langur retrovirus (LRT) major capsid protein and goat antiserum to squirrel monkey retrovirus (SMRV) also has been described (Kajikawa *et al*. 1990); the use of heterologous reagents and radioisotopes did not make the use of this technique more advantageous.

The sequencing of one strain of JSRV (York *et al*, 1992) offered new possibilities for research through the generation of JSRV recombinant antigens and homologous antisera.

This chapter describes the generation of new immunological reagents, the development of a blocking enzyme-linked immunosorbent assay (B-ELISA) and an immunohistochemical technique for the detection of JSRV major capsid protein at tissue and cellular levels. Studies were then undertaken to examine the distribution

and sites of replication of JSRV in SPA-affected and unaffected sheep to better define the role of this virus in the pathogenesis of the disease.

3.2 Materials and Methods.

The constructs expressing the recombinant JSRV-CA proteins, the homologous rabbit antiserum and the protocols to purify JSRV-CA recombinant proteins were available at Moredun Research Institute before the start of this thesis as result of the work of Ms. P. Dewar and Mr. N. F. Inglis. A brief description on how these reagents have been produced is included in 3.2.1 and 3.2.2 for a better understanding of this chapter.

3.2.1 Subcloning and expression of clone JS382.

Plasmid pBluescript-Js382 containing a part of the JSRV *gag* gene (bases 953 to 3030 of the nucleotide sequence published by York *et al*, 1992) was a gift from Dr. G. Quérat (Marseilles). Briefly, the insert fragment was excised by *EcoRI* restriction, subcloned into plasmids pMS1S (Sherf *et al*. 1990, supplied by M. Shreiber) and pGEX1 λ T (Pharmacia) and expressed in *E. coli* host strain NM522 as β -galactosidase (β gal-CA, plasmid pMCA) and glutathione-S-transferase (GST-CA, plasmid pGCA) fusion proteins respectively.

Confirmation that the *gag* gene was in the correct reading frame was obtained by sequencing across the vector-insert junction, as well testing clones for production of β -galactosidase and GST fusion proteins of the appropriate size by western blotting (immunoblot) analysis with a goat antiserum to Mason-Pfizer monkey virus major capsid protein (MPMV-CA) (ref. 75S - 148, National Cancer Institute Repository) (Sharp and Herring, 1983).

Transformed bacteria were grown and induced with IPTG for the expression of recombinant proteins.

Bacteria were pelleted (5000 x g for 10 min) and resuspended in 20 ml TE (10mM Tris pH7.5, 1mM EDTA). Phenylmethylsulphonylfluoride (2 mM) was added before lysing the cell suspension in a French press at 1500 psi (10.35 MPa). The lysate obtained was sonicated and clarified at 100000 x g at 4°C for 10 min.

βgal-CA fusion protein was purified by affinity chromatography using a 4 ml column of aminobenzyl-1-thio-β-galactopyranoside (ABTG) agarose (Sigma). The column was washed with 20 ml of 300 mM NaCl-50mM Tris/HCl pH 8, 10 ml of 600 mM NaCl - 50 mM Tris/HCl pH8, and 20 ml of 50 mM Tris/HCl pH8 and the βgal-CA eluted with 20 ml of 100 mM sodium borate pH 10 and collected on ice into a tube containing 1 ml of 2M tris-HCl pH 7. The eluate was dialyzed against PBS overnight at 4°C and then concentrated to 2-3 ml with PEG 20000 (Cameron *et al*, 1994). The yield of soluble βgal-CA was approximately 9 mg/l of bacterial culture. Protein concentration was estimated using the BCA™ Protein Estimation Assay (Pierce), as recommended by the manufacturer.

GST-CA was purified by affinity chromatography using a 4 ml column of glutathione sepharose (Pharmacia) as recommended by the manufacturers.

GST-CA could not be eluted with free glutathione from the sepharose beads as recommended by the manufacturers and therefore rabbits were immunised with the fusion protein coupled to the beads.

3.2.2 Production of rabbit polyclonal antiserum to JSRV-CA.

A specific rabbit antiserum to JSRV-CA was prepared by immunising rabbits with 500 µg βgal-CA combined with Freund's incomplete adjuvant. After 15 days the rabbit was boosted with 500 µg of GST-CA bound to the glutathione sepharose beads in the absence of adjuvant. A third injection of GST-CA (500 µg) was given after 4 weeks and the rabbit was bled 15 days after the last injection.

To avoid non-specific reactions in the B-ELISA and in the immunohistochemical study, the antiserum obtained was absorbed overnight at 4°C in a rotating wheel with a lysate of IPTG-induced NM522-pMS1S cells (400 µl serum + 15.6 ml washing

fluid B + 4 ml lysate). The serum was then centrifuged at 100000 x g for 30 min to pellet any bacterial debris, aliquoted and stored at -20°C until used.

3.2.3 Preparation of samples tested.

To determine the best conditions for the B-ELISA, to assess its specificity and its inter- and intra-assay standard deviation the following samples were employed:

i) two different pools of lung fluid (LFP#1 and 2) collected from SPA sheep.

LFP#1 was filtered through a double layer of gauze and clarified by centrifugation at 10000 x g for 1h at 4°C. The resultant supernatant was aliquoted and stored at -70 °C until use. Part of the supernatant (60 ml) was further ultracentrifuged at 100000 x g through a double layer of glycerol (25/50% v/v) for 1h at 4°C and the resultant pellet was resuspended in 150 µl of TNE. A 10 µl aliquot was checked for the presence of JSRV by SDS-PAGE/ western blotting employing the rabbit antiserum to JSRV-CA and a goat antiserum to MPMV-CA (as described in 3.2.5).

LFP#2 was filtered, clarified and ultracentrifuged as above. An aliquot of the supernatant was stored at -70°C before and after the ultracentrifugation step. The pellet obtained by ultracentrifugation was resuspended in TNE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA) and further purified by isopycnic centrifugation (100000 x g for 16h at 4°C) on 20 to 55% (w/w) continuous sucrose gradient. The gradient was fractionated and each 0.5 ml fraction was resuspended in 4.5 ml TNE buffer, centrifuged at 100000 x g for 1h at 4°C and the resultant pellet resuspended in 100 µl TNE.

ii) A pool of lung washings collected from healthy sheep.

Lung washings were obtained immediately after the lungs were excised from the thoracic cavity of the sheep at post-mortem examination by introducing 250-500 ml of TNE into the trachea, the lungs were then gently massaged and the washings were finally collected in a sterile beaker. The washings were then filtered through a double layer of sterile gauze, clarified by centrifugation at 10000 x g for 1h at 4°C and ultracentrifuged at 100000 x g for 1h at 4°C through a double layer of glycerol

(25/50% v/v). The resultant pellet was resuspended in TNE as a 400 times concentrate, aliquoted and stored at -70°C.

iii) Sucrose gradient purified maedi-visna virus (MVV; gift of Dr. R.G. Dalziel, Edinburgh);

iv) A lysate of NM522-pMS1S cells induced with IPTG to provide a β -galactosidase control.

v) A lysate of a NM522-pGex1 λ T cells induced with IPTG to provide a GST control.

For analysis of the distribution of JSRV in affected and unaffected animals, samples were collected from:

a) 9 sheep naturally affected by SPA;

b) 16 lambs, experimentally infected as described in 2.2.;

c) 16 age and breed-matched unaffected sheep.

Samples of lung, lung tumour, mediastinal and retropharyngeal lymph nodes, thymus, tonsils, Peyer's patches, spleen, bone marrow, kidney and lung washings were collected during the post-mortem examination and snap frozen in liquid nitrogen before storage at -70°C. Lung fluid and heparinised blood samples were taken immediately before the sheep were killed. In addition, tumour samples collected in Italy from three goats which died from enzootic nasal tumour were also tested. Lung fluid samples were filtered, clarified at 10000 x g for 1h at 4°C and the resultant supernatants were stored at -70°C. Lung washings from control animals were collected and processed as described above in point (i). Plasma samples (8 ml per sample) were clarified as above and further centrifuged at 100,000 x g / 1h / 4°C and the pellets resuspended in 65 μ l of TNE before storing at -70°C until use. Leukocytes were obtained from blood samples in which the red cells had been lysed with a hypo-osmotic solution (144 mM NH₄; 17 mM Tris, pH 7.65). Each leukocyte sample (7 x 10⁷ cells) was diluted in TNE, freeze/thawed three times, and clarified and centrifuged as described above. After the ultracentrifugation step, pellets were resuspended in 65 μ l of TNE and stored at -70°C.

Tissues samples were homogenised (10% w/v suspension in TNE buffer) with a pestle and mortar, filtered, clarified and ultracentrifuged as above. All tissue samples were concentrated 400 times except tonsils which were concentrated only 100 times due to the small size of this organ. One hundred μ l of bone marrow samples extracts were further purified by isopycnic centrifugation in sucrose gradients as described for LFP#2 above in point (ii). Tissues extracts were kept at -70°C until further use.

3.2.4 Development of a blocking enzyme linked immunosorbent assay (B-ELISA).

The SPA B-ELISA depends on the presence of JSRV in the test samples to inhibit the binding between purified β gal-CA and the rabbit antiserum to JSRV-CA. A schematic diagram of the B-ELISA is represented in Fig. 3.1 and 3.2.

Equal volumes of the test sample and WFB were reacted at room temperature for 1 hour in order to disrupt the envelope of JSRV and release the capsid of the virions. WFB was also used as washing buffer for the ELISA plates and as dilution buffer for the reagents of the reaction.

Microtitre plates were coated with 100 μ l of purified β gal-CA (1.5 μ g/ml), diluted in carbonate buffer (pH 9.6), by incubating overnight at 4°C in a humid chamber (Fig. 3.1[1] and 3.2 [1]). After incubation, the plates were washed three times with WFB and blocked by incubation for 1h at 37°C with a 1% (w/v) solution of bovine serum albumin in PBS (Fig 3.1 [2] and 3.2 [2]).

The antiserum and test samples were reacted first in a V-bottom “transfer” plate (65 μ l of 1:800 dilution of rabbit antiserum to JSRV-CA + 65 μ l of test samples pre-treated with WFB) for one hour at 37°C (Fig 3.1[1a] and 3.2 [1a]). Thereafter, 100 μ l of the resulting mixture (rabbit serum + sample) were transferred to the pre-coated ELISA plates, which had been washed three times with WFB (Fig 3.1[3] and 3.2[3]).

The ELISA plates were incubated at 37°C for 1h and, after washing three times, 100 μ l of pig antiserum to rabbit IgG conjugated to horse radish peroxidase (DAKO -

1:1000 in WFB) was added to each well (Fig 3.1[4] and 3.2[4]). After incubation at 37°C for 1h, the plates were washed four times with WFB and 100µl of substrate (orthophenyldiamine, Sigma) + 0.05% H₂O₂ (30% v/v) were added to each well (Fig. 3.1[5] and 3.2[5]). The colour development was stopped with 2.5M H₂SO₄ and the plates read using a Dynatech MR5000 apparatus at 490 nm.

Samples were tested in duplicate in each test. Controls included wells with no inhibitor (0% blocking) and wells with neither inhibitor nor serum (100% blocking) the missing component being replaced with diluent (WFB).

Results are expressed as a percentage blocking value using the following formula:

$$\% \text{blocking value} = [(OD_0 - OD_{\text{test}}) / (OD_0 - OD_{100})] \times 100$$

Where OD₀ is the OD at 0% blocking control , OD₁₀₀ is the OD at 100% blocking control and OD_{test} is the OD of the test sample.

The optimal concentrations of βgal-CA and the rabbit antiserum were predetermined by a checkerboard analysis. Two ELISA plates were coated with increasing amounts of βgal-CA (500 ng to 3 µg). Then, two parallel reactions were performed where doubling dilutions of the rabbit antiserum (1:100 to 1:3200) were reacted either with LFP#1 (positive control) or with a pool of lung washings collected from healthy sheep (negative control). The optimal concentration of both βgal-CA and rabbit antiserum was the combination which gave the greatest difference in the blocking values between the positive and the negative controls.

The best format for the test was also pre-determined, considering that the test can be done in two ways, namely, simultaneous addition of test sample and rabbit anti JSRV-CA serum to the pre-coated ELISA plate leading to a “competitive” ELISA, or pre-incubation of the test sample and the rabbit serum prior to addition to the ELISA plate (“blocking” ELISA). Comparison between B-ELISA and competitive ELISA was performed by assessing duplicates of LFP#1 and negative controls (lung washings from healthy sheep) in the two different formats.

Elisa plate

Transfer plate

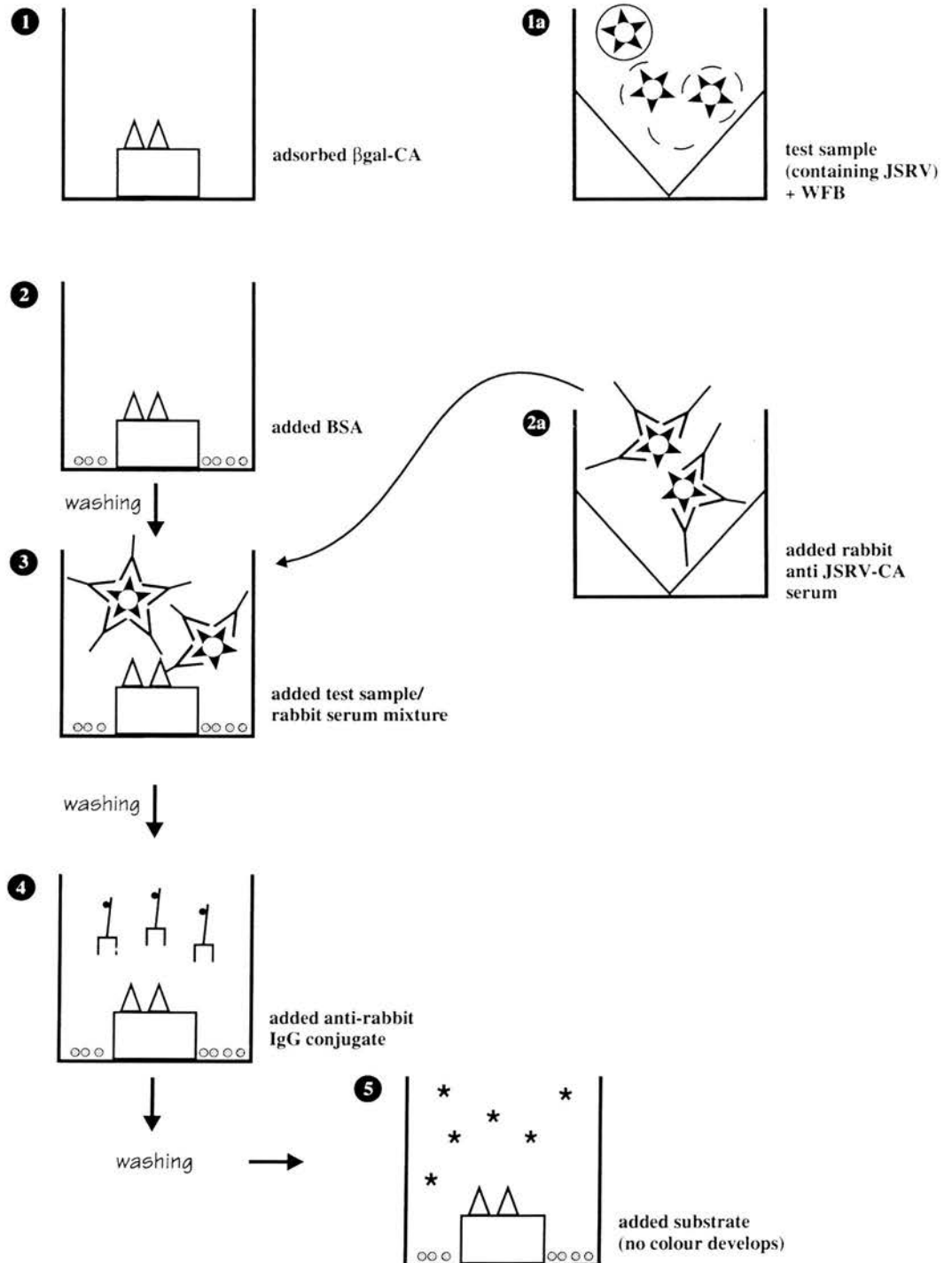


Fig 3.1: Schematic diagram of the SPA B-Elisa. In this case the test sample contains JSRV virions; note the lack of the colorimetric reaction after the addition of the substrate (step 5). See text for details.

Elisa plate

Transfer plate

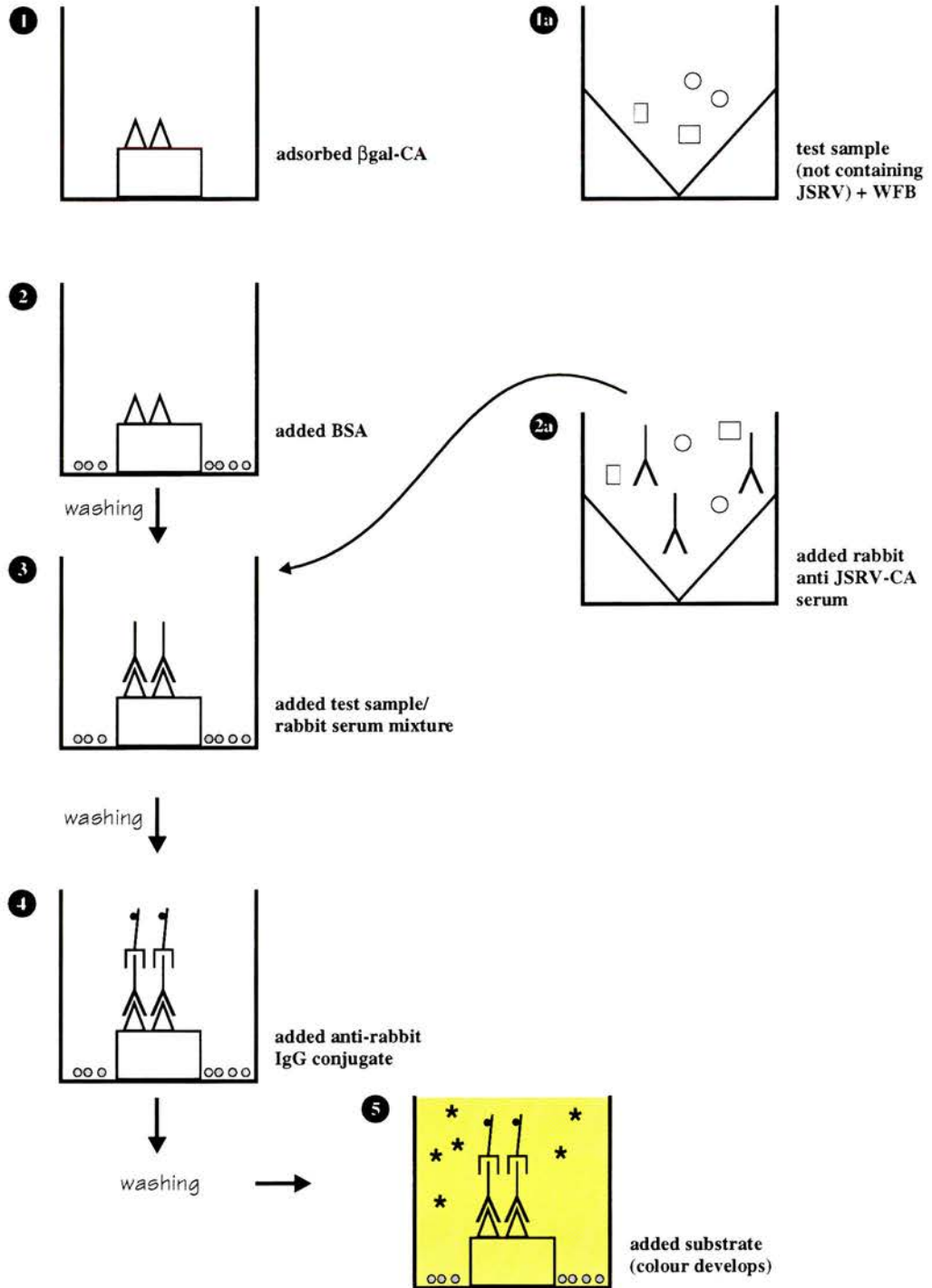


Fig 3.2: Schematic diagram of the SPA B-Elisa. In this case the test sample does not contain JSRV virions; note the presence of the colorimetric reaction (represented in yellow) after the addition of the substrate (step 5). See text for details.

The intra-assay standard deviation was evaluated testing LFP#1 across the ELISA plate in five different plates and calculating the mean between the standard deviations obtained in each plate. The inter-assay standard deviation was calculated by assaying LFP#1 in ten independent tests performed on different days. LFP#1 was located in the same wells on the ELISA plate on each occasion.

3.2.5 SDS Polyacrylamide Gel Electrophoresis and Western blotting.

Proteins were separated on SDS gels essentially as described by Laemmli (1970). Briefly, samples were denatured by heating at 100 °C for 90 sec in an equal volume of x2 Laemmli sample buffer (0.5 M Tris/HCl pH 6.8, 4% SDS, 8% β -mercaptoethanol, 20% [w/v] sucrose, 0.002% [w/v] bromophenol blue). Samples were then loaded into the wells of an SDS PAGE gel comprising a 3% stacking gel (3% [w/v] acrylamide, 125 mM Tris/HCl, 3.5 mM SDS pH 6.8) and a 10% resolving gel (10% [w/v] acrylamide, 375 mM Tris/HCl, 5 mM SDS pH 8.8) in Laemmli electrode buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS pH 8.3). The gels were run at a constant 200 V for 30-40 min using a Bio-Rad Mini Protean II™.

To immunologically characterise the new reagents developed, western blotting was performed on the purified β gal-CA, on LFP#1 and on LFP#2 sucrose gradient purified fractions employing a goat antiserum to MPMV-CA and the rabbit antiserum to JSRV-CA as described previously (Sharp & Herring, 1983).

Briefly samples were transferred from SDS PAGE gels onto nitrocellulose membranes (Bio-Rad) by electroblotting in Tris/glycine blot buffer (25mM Tris, 192 mM glycine, 20% [v/v] methanol, pH 8.3), at a constant 100 V for 1 h. After staining with Ponceau red to confirm transfer of the separated proteins, the membrane was washed three times in WFB. Antisera, goat anti MPMV-CA diluted 1:40 in WFB, or the rabbit anti JSRV-CA diluted 1:200 in WFB, were added and incubated for 1 h at room temperature. After washing three times, rabbit anti-sheep or swine anti-rabbit horse radish peroxidase conjugate diluted 1:1000 in WFB was added as appropriate and incubated for a further hour at room temperature. After further washings, the

reaction was visualised with diaminobenzidine as substrate (100 mM Tris/HCl pH 7.5, 0.15% (v/v) H₂O₂, 0.2% (w/v) diaminobenzidine [DAB, Sigma]).

Ten µl aliquots of bone marrow extracts prepared as described in 3.2.3 also were tested by SDS-PAGE western blotting employing the rabbit anti JSRV-CA serum as above.

3.2.6 Immunohistochemistry.

Tissue samples used in immunohistochemistry studies were collected during the post-mortem examination from four SPA-affected sheep (three experimentally infected lambs and one natural case of SPA) and three uninfected controls. Portions of the same tissues that were collected for the B-ELISA were also fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned at 4-6 µm. Selected sections were stained immunohistochemically using a commercial avidin-biotin peroxidase complex kit (Vectastain ABC kit™ -Vector laboratories) essentially as recommended by the manufacturers. Sections were incubated at 60°C for 30 min and then deparaffinized with xylene 3 times for 20 min and then hydrated through a series of alcohol solutions (100% to 60%, v/v). After brief rinsing in tap and distilled water, sections were treated with 1% (v/v) hydrogen peroxidase in methanol for 30 min at room temperature to inactivate endogenous peroxidases. Sections were washed again in tap water for 10 min, two times in distilled water for 5 min at room temperature, once with TBS (100 mM NaCl, 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5) at room temperature and twice at 37°C. Slides were then incubated at 37°C for 25 min with a trypsin solution (solution 0.1% trypsin[w/v], 0.1% Cl₂Ca in TBS[w/v], pH 7.8) and washed afterwards as above. Possible non-specific binding of the conjugate used (see below) was avoided by incubating the sections with normal goat serum (1:20 in TBS) in a humid chamber for one hour at 37°C. Normal goat serum was then removed by inverting the slides without further washings. Slides were then incubated in a humid chamber with the rabbit anti JSRV-CA serum (at the optimal dilution of

1:50) overnight at 4°C. As a control, this serum was substituted with TBS to detect any endogenous peroxidase activity of the tissues or with rabbit pre-immunisation serum to check for non-specific reactions of the rabbit serum. Sections were again washed 3 x 10 min with TBS/Tween (TBS, 0.5% [v/v] Tween) and incubated with a biotinylated goat anti rabbit immunoglobulin (1:100; Vector Laboratories). After three washings of 10 min each with TBS/Tween, the ABC Vectastain complex™ containing avidin and biotinylated horse radish peroxidase was added to the sections and incubated for one hour at room temperature. Washings were then performed as above and finally the reaction was visualised by incubation of the sections with DAB substrate solution (0.05% DAB [w/v] in TBS, activated with 0.15% [v/v] H₂O₂). After one minute, the reaction was stopped with tap water and then the sections were counterstained with Carazzi's haematoxylin, dehydrated and mounted with a coverslip.

3.3 Results.

3.3.1 Development and validation of the JSRV B-ELISA.

- *Specificity of reagents*

Both the rabbit anti-JSRV-CA (Fig. 3.3a) and the goat anti MPMV-CA (results not shown) recognised β gal-CA and a single polypeptide of about 25 kDa in LFP#1 (Fig. 3.3b).

- *The %blocking values of the B-ELISA are the result of the interaction between JSRV particles and the specific rabbit antiserum produced.*

A series of experiments were performed to demonstrate that the blocking values obtained in the B-ELISA were the result of the interaction between JSRV-CA and the specific rabbit antiserum produced in this study and not due to non-specific reactions.

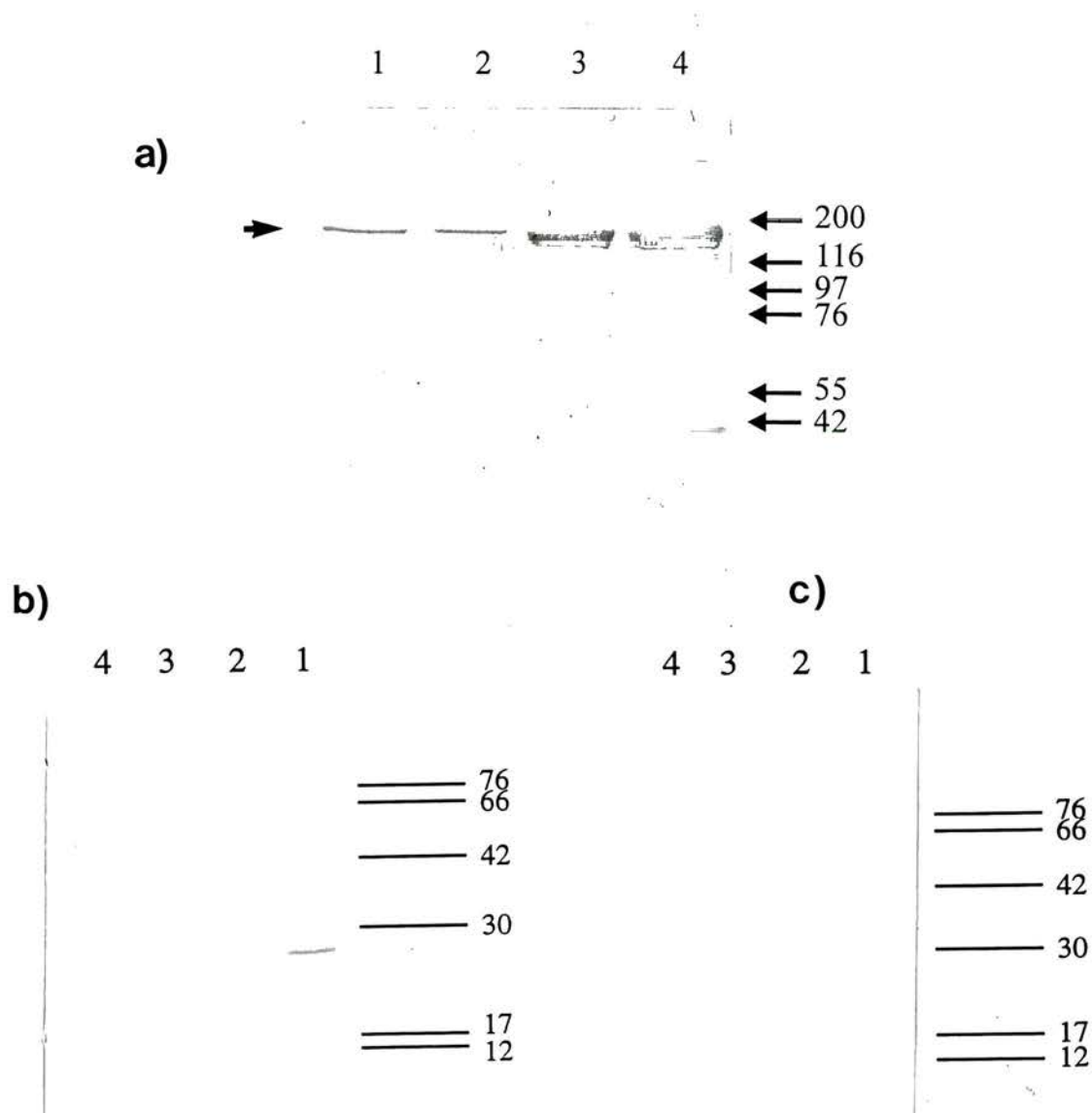


Fig. 3.3: **Specificity of the rabbit serum antiserum to JSRV-CA.** (a) Western blot showing the detection of purified β gal-CA (expected molecular weight = 188 kDa) with the rabbit antiserum to JSRV-CA. Lanes 4-2, ABTG column flow through after washings with 300 mM NaCl-50mM Tris/HCl pH 8 (lane 4), 600 mM NaCl -50 mM Tris/HCl pH8 (lane 3) and 50 mM Tris/HCl pH8 (lane 2); lane 1, eluted β gal-CA (arrow). Molecular weight marker is indicated (BDH, 42,700 - 200,000). (b): The same antiserum reacts with a polypeptide of about 25 kDa in LFP#1; lane 1-4 = two fold serial dilutions of ultracentrifuged LFP#1 prepared as described in point (i) of 3.2.3. In sucrose-gradient purified JSRV (c) it is possible to note the reaction of the rabbit antiserum to JSRV-CA with a single polypeptide of approximately 25 kDa; lane 1, fraction with 1.171 (g/cm^3) density; lane 2, fraction with 1.181 density; lane 3, fraction with 1.186 density; lane 4, fraction with 1.197 density; molecular weight marker is indicated (BDH, 12,300 - 78,000).

Analysis of the 18 fractions of LFP#2 separated by isopycnic centrifugation showed that the greatest %blocking values corresponded to fractions having a buoyant density between 1.16g/ml to 1.19g/ml, with a peak at 1.18g/ml which is the buoyant density of JSRV (Sharp and Herring, 1983) (Fig. 3.4). The same fractions analysed by western blotting, employing the rabbit antiserum to JSRV-CA produced in this study showed a single polypeptide of about 25,000 M_r , the predicted molecular weight of JSRV-CA (3.3c).

The blocking value of LFP#2 before ultracentrifugation was 63% which was reduced to only 10% for the post-centrifugation supernatant, demonstrating that the blocking activity was particle-associated, and did not arise as a result of antibodies specific for JSRV-CA that might have been present in the lung fluid. In other experiments, maedi-visna virus, another retrovirus of sheep which is occasionally found to coinfect SPA affected animals (see 1.9), the β -galactosidase control and the GST control all gave %blocking values less than 7.

These data demonstrate that the blocking value of the SPA B-ELISA depends on the presence in the test samples of the major capsid protein of JSRV.

- *Test format, intra-assay and inter-assay standard deviation..*

The B-ELISA format was chosen because it was found more sensitive than the competitive format. The %blocking value of ultracentrifuged LFP#1 was 81 in the blocking ELISA format and 73 in the competitive format; the negative control %blocking value was respectively 8 and 9. The intra-assay standard deviation was $\pm 1.6\%$ while the inter-assay standard deviation was $\pm 4.6\%$.

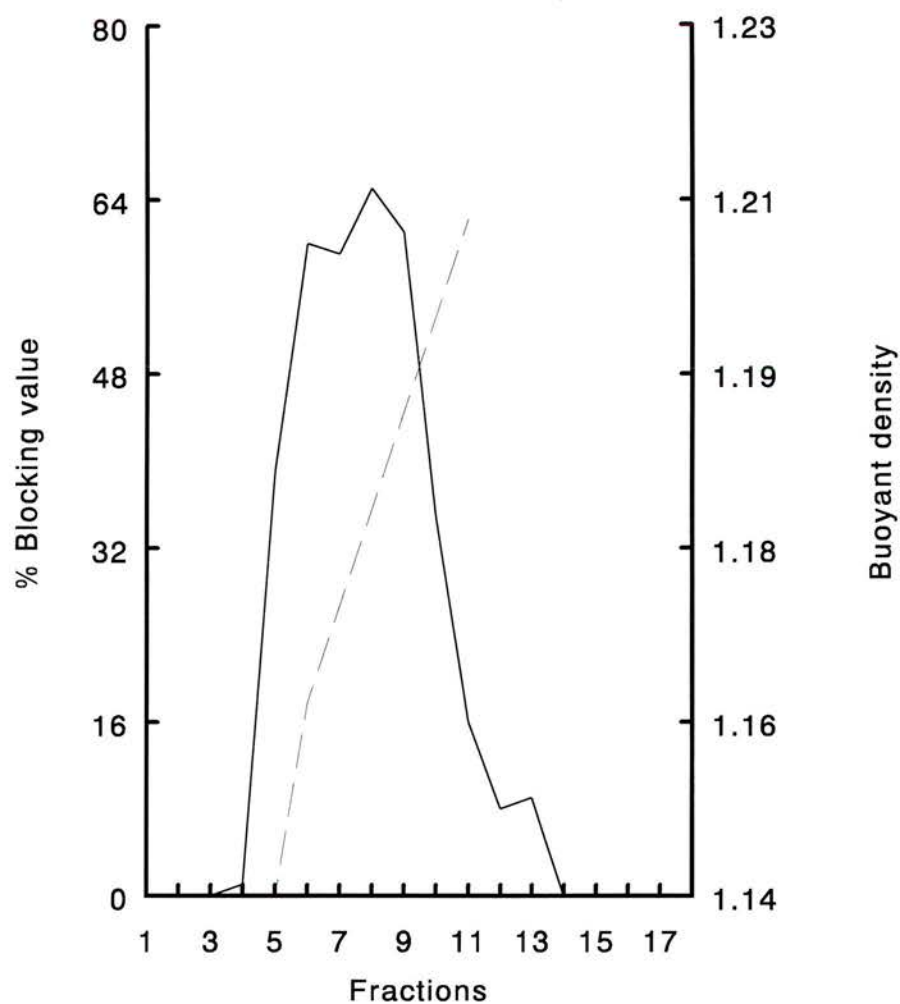


Fig. 3.4: Percent blocking value of LFP#2 after isopycnic centrifugation in 20-55% (w/w) sucrose gradients. The continuous line shows the %blocking values; the broken line shows the density (g/ml) after sucrose gradient centrifugation.

3.3.2 Distribution of JSRV viral particles in tissue extracts of SPA-affected and unaffected sheep.

Following the successful development of the B-ELISA to detect JSRV-CA, this assay was used to examine the anatomical distribution of JSRV in sheep. The samples tested were obtained by ultracentrifugation and therefore reflected the presence of JSRV viral particles.

Statistical analysis of the differences between SPA-affected and unaffected animals was undertaken using the student t-test in the Minitab™ statistical computer package. Results are summarised in Table 3.1.

Only tumour and lung samples showed a significant difference between affected and unaffected sheep ($p < 0.0001$). The mean %blocking value of tumour samples was 77.8 with values ranging from 41 to 93 while normal lung samples had a mean %blocking value of 17 with a range from 0 to 33.

A %blocking value of 36 was selected as the highest negative value calculated from the mean blocking value of control lungs plus 2.5 standard deviations. When this value was used no false positive or false negative samples were observed.

No significant difference was observed between the mean blocking values obtained using lung tumour samples collected from natural or from experimentally induced cases of SPA. Lung fluid samples from SPA cases showed a high %blocking value (mean = 68.4) with values ranging from 49 to 92 but because healthy sheep do not produce collectable quantities of lung fluid no direct control for this sample could be obtained. The closest control for the lung fluid samples was broncho-alveolar lavages from unaffected sheep (lung washings), which gave values ranging from 1.5 to 20.

In all the other tissues examined there was no statistically significant difference between SPA-affected and unaffected sheep with the exception of a single mediastinal lymph node sample, collected from a natural case of SPA. This sample had a %blocking value of 53, markedly greater than the mean %blocking value of equivalent tissues in control animals (6.2). Bone marrow samples showed a high mean blocking value in both affected and unaffected controls. The mean %blocking

values were 40.2 for SPA sheep and 45.2 for the unaffected controls. These high %blocking values were probably an artefact due to the nature of the sample after processing. The preparation of this tissue, as described in Materials and Methods, resulted in a viscous pellet which therefore could have blocked the reaction non-specifically. Further dilutions or sucrose gradient purified samples resulted in the almost complete loss of blocking activity (< 16) and no specific reaction in this tissue was found by immunohistochemistry (see below) or western blotting.

The mean %blocking value of the goat nasal tumour extracts was 74 (± 3.5). This result confirmed the strict similarity between ENTV and JSRV.

3.3.3 Cellular localisation of JSRV-CA.

Immunohistochemical analysis using the rabbit antiserum to JSRV-CA showed an intense diffuse brown staining, due to the precipitation of the DAB, in the cytoplasm of recognisable alveolar neoplastic cells in all four SPA lungs examined (Figs. 3.5 and 3.6). No staining was observed in the controls with pre-immune rabbit serum or with TBS.

In the same lesion, not all the tumour cells were equally stained and some were not stained at all (Fig. 3.5 [a], [b] and 3.6[c]). There was no staining of the epithelial non-transformed cells (3.5[b]) nor of the interstitial cells (3.5[c]). Staining in the extracellular airway spaces was observed very rarely.

No specific staining was detected in the lungs of control unaffected sheep (Fig. 3.6[a]), nor in any other tissue from both SPA-affected sheep and unaffected control animals. An intracytoplasmatic granular staining was observed in the thymus samples of both affected and non-affected animals. However this staining proved to be non-specific because it was also present when the rabbit pre-immunisation serum was used instead of the rabbit anti-JSRV-CA (Fig. 3.5 [d-e]).

Table 3.1. Results of the B-ELISA on tissues of SPA affected and unaffected sheep.

| <i>SAMPLES</i> | <i>SPA+*</i> | | <i>SPA-†</i> | |
|-----------------------------|-----------------------------------|-----------|-----------------------------------|----------|
| | <i>%blocking value(±s.e.)</i> | <i>n‡</i> | <i>%blocking value(±s.e.)</i> | <i>n</i> |
| Lung tumour/ Lung | 77.8 (±4.8)§ | 13 | 17 (±1.8) | 13 |
| Lung fluid | 68.4(±3.3) | 15 | 9.9 (±2.1)@ | 11 |
| Mediastinal lymph nodes | 15.2 (±3.7) | 12 | 6.2 (±2.1) | 8 |
| Retropharyngeal lymph nodes | 6.6 (±1.4) | 8 | 7.0 (±2.4) | 7 |
| Tonsils | 4.7 (±2.3) | 9 | 3.7 (±1.5) | 9 |
| Bone marrow | 40.2 (±4.06) | 6 | 45.2 (±5.7) | 6 |
| Thymus | 18.7 (±3.8) | 7 | 13.1 (±2.7) | 13 |
| Peyer's patches | 12.6 (±1) | 5 | 7.8 (±2.7) | 6 |
| Spleen | 18.7 (±2.7) | 9 | 16.1 (±3.9) | 8 |
| Kidney | 6.5 (±2.9) | 8 | 8.3 (±4.1) | 7 |
| Plasma | 10.8 (±3) | 10 | 7.6 (±3.1) | 5 |
| Leukocytes | 12.4 (±2.2) | 10 | 13.4(±1.1) | 5 |

*= SPA-affected animals

†= unaffected controls

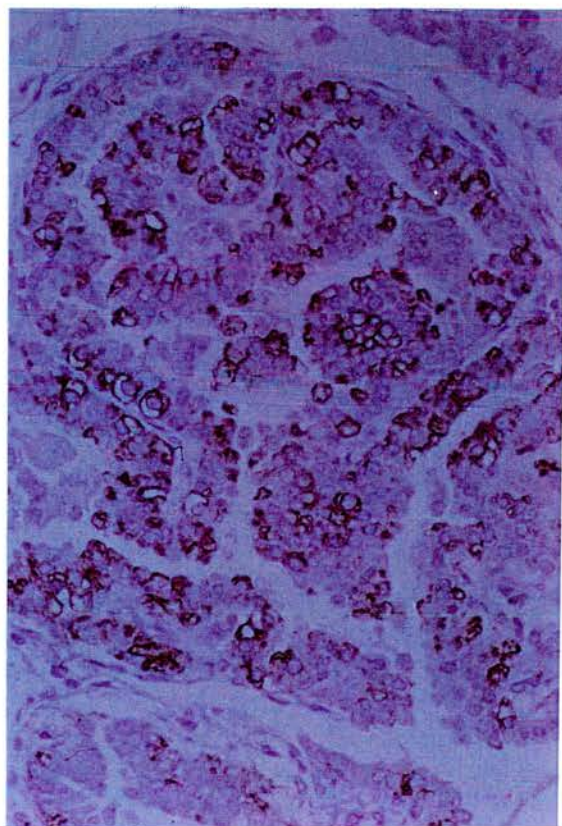
‡= number of samples tested

§= standard error of the mean

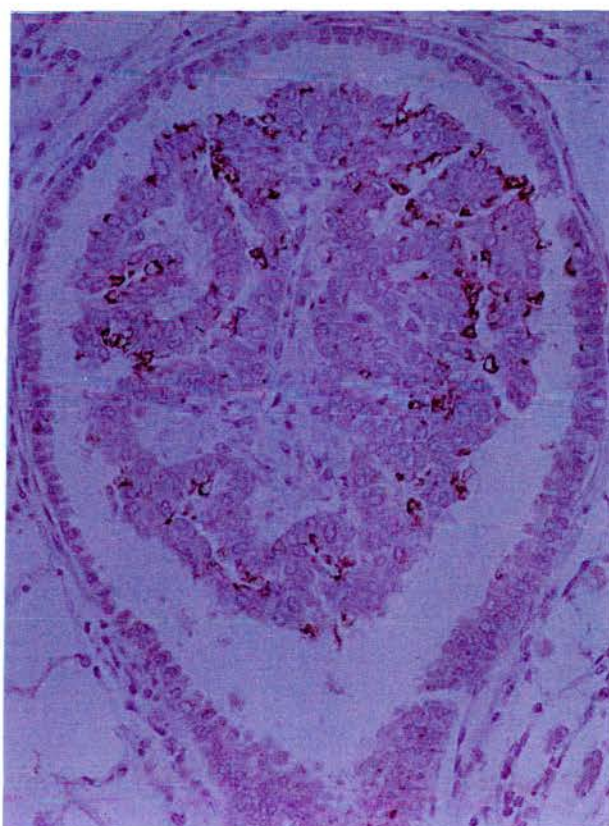
@= this value is referred to lung washings.

Fig. 3.5: **Immunohistochemistry**; (a-c) show lung tumour sections of a sheep affected by SPA. The brown staining represents the positive reaction and is confined to the cytoplasm of the transformed cells and is clearly visible in (a)(magnification 250 x) and (c) (magnification 400 x). A papillary projection of the tumour in the bronchiolus is visible in (b) (magnification 250 x). The brown staining is confined to the neoplastic cells although not all the tumour cells are stained, as can be seen in (b). No staining is observed in the interstitial cells or in the untransformed alveolar and bronchiolar cells (b-c). Non-specific immunostaining in a thymus section of a SPA-affected animal is visible in (d) and (e) (magnification 250 x). A granular brown staining is visible in the cytoplasm of some cells when stained with either rabbit anti JSRV-CA serum (d) or pre-immune rabbit serum (e).

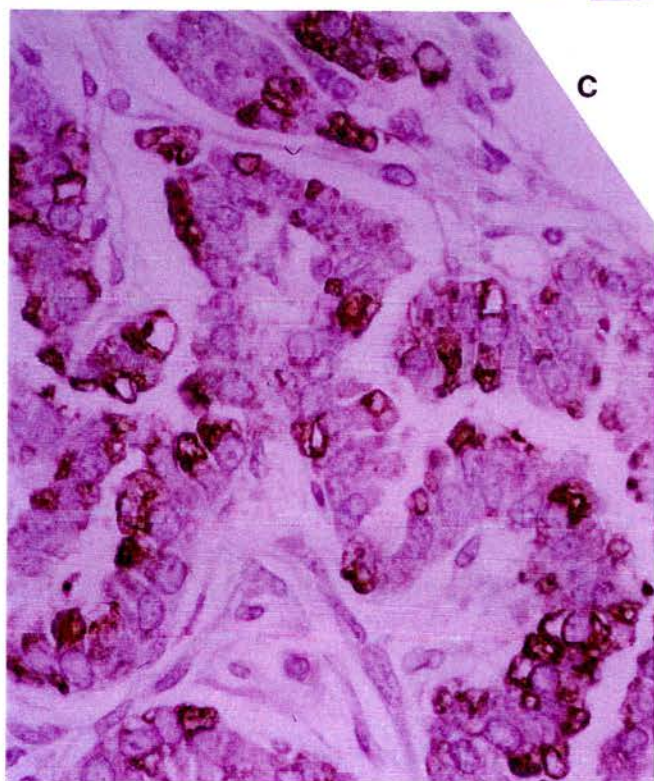
a



b



c



d



e

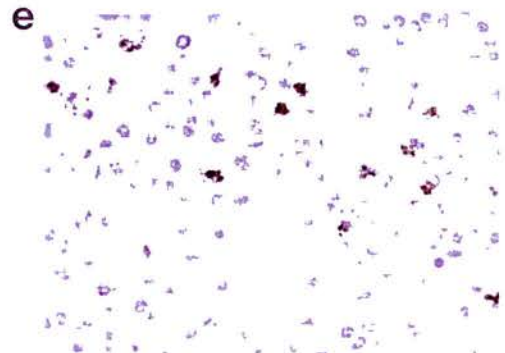
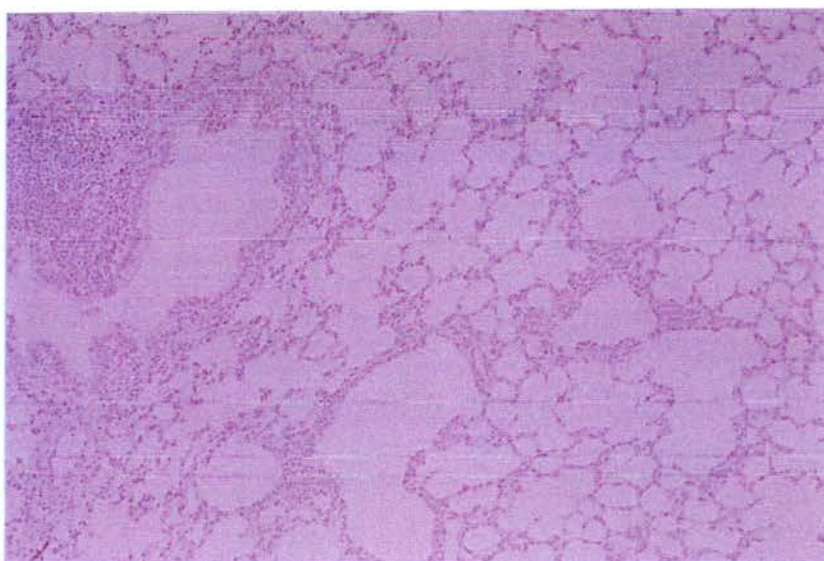
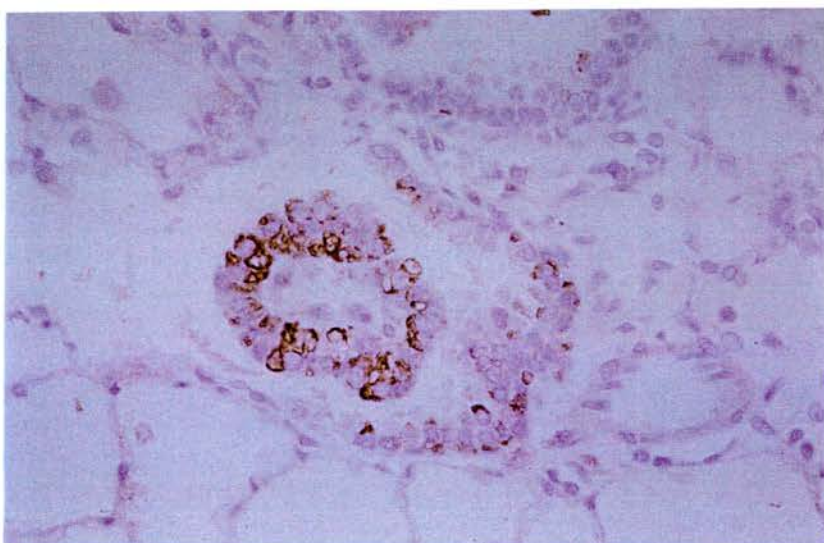


Fig. 3.6: **Immunohistochemistry**; (a) shows a lung section of a healthy control sheep (magnification 100 x). No staining is visible in the section while staining is visible in early SPA lesions in (b) (magnification 250 x) and (c) (magnification 200 x).

a



b



c



3.4 Discussion .

The results of this study using immunological techniques have shown that JSRV virions and CA were detected only in the respiratory tract of sheep affected by SPA. These findings have confirmed earlier reports describing type D retrovirus antigens or reverse transcriptase activity in respiratory tissues of SPA sheep (Perk *et al*, 1974; Martin *et al*, 1976; Herring *et al*, 1983; Sharp & Herring, 1983; Verwoerd *et al*, 1983; Rosadio *et al*, 1988) and extended these observations by demonstrating that JSRV was not detected in 172 samples from eight other tissues from 41 sheep, although viral expression, below the detection limit of the B-ELISA, may be occurring in other tissues.

Immunohistochemistry supported the above conclusion and further indicated that JSRV replication occurred primarily in the transformed epithelial cells in the pulmonary alveoli of SPA sheep. A notable exception was the positive result obtained with a single lymph node from a field case of SPA that could have arisen either as a consequence of metastatic events, which are known to occur in a proportion of cases (Nobel *et al*, 1969), or as an occasional spread of JSRV in the regional lymph nodes.

Previous immunohistochemical studies employing a heterologous antiserum to MPMV-CA detected putative JSRV antigens in the alveolar lumina (Payne *et al*, 1986), rather than intracellularly and therefore conflict with those reported here. However, the present study has used an homologous antiserum and the results obtained are in agreement with earlier ultrastructural studies describing intracytoplasmic type A particles in the epithelial tumour cells and, associated with these cells, a few extracellular particles with typical type D morphology (Perk *et al*, 1971; Sharp *et al*, 1983; Payne *et al*, 1983).

The above findings point to the epithelial tumour cells in the alveoli as the major site of translation and assembly of JSRV and highlight the strict association between JSRV and the tumour. These findings, therefore, provide further support for the notion that JSRV is the aetiological agent of SPA. Although most type D retroviruses

appear not to be oncogenic, a simian type D retrovirus (SRV 2) has been associated aetiologically with retroperitoneal fibromatosis in macaques (Bryant *et al*, 1986) and the other sheep and goat type D-related viruses have been implicated in tumours of the nasal mucosa (De las Heras *et al*, 1991b, 1993 & 1995; Vitellozzi *et al*, 1993). As expected ENTV cross-reacted with the rabbit antiserum to JSRV-CA demonstrating a close relationship between these two viruses.

Whilst the circumstantial evidence implicating JSRV in neoplasia is compelling, the existing information does not rule out other possibilities in which it may, or may not, be involved in the neoplastic processes. JSRV may be acting only as a helper for some other replication-defective, acutely transforming retrovirus or result from reactivation of endogenous viral sequences as a consequence of neoplasia, as seen with other endogenous retroviruses (see 1.6.3). The presence in the sheep genome of up to 15-20 copies of sequences related to the *gag*, *pol* and *env* genes of JSRV (York *et al*, 1992; Hecht *et al*, 1994) introduces major complications, particularly as endogenous retroviruses have been demonstrated in mice, chickens and cats to be involved in recombination events between each other or with their exogenous counterparts (Weiss *et al*, 1973; Hartley *et al*, 1977; Golovkina *et al*, 1994; McDougall *et al*, 1994; Bai *et al*, 1995).

In the next two chapters further studies aimed at determining the relationship between the exogenous and endogenous viruses will be described.

Chapter 4.

Distinction between JSRV and JSRV-Related Endogenous Retroviruses (*enJSRVs*).

4.1 Introduction.

In Chapter 3 it was established that epithelial tumour cells in the lungs of sheep affected by SPA are the major sites of JSRV replication.

The working hypothesis of this project is that JSRV is an horizontally transmitted virus specifically associated with SPA. However, the presence of endogenous JSRV-related sequences (*enJSRVs*) in the sheep genome makes possible at least two different scenarios: i) JSRV is an horizontally transmitted virus distinct from its endogenous counterparts; ii) JSRV derives from the reactivation of one or more endogenous retroviruses under pre- or post-neoplastic stimuli.

Little information is available about *enJSRVs* sequences. Studies conducted by Southern hybridisation with JSRV *gag*, *pol*, or *env* probes showed the presence of 15 to 20 copies of cross-reacting bands in sheep genomic DNA (York *et al*, 1992; Hecht *et al*, 1994). The degree of homology between JSRV and *enJSRVs* must be very high because the filters in the Southern analysis were washed under high stringency conditions.

In order to undertake any study at the molecular level, it was therefore necessary to establish a difference (if this difference existed) between the exogenous JSRV, apparently present only in tumours of sheep affected by SPA, and *enJSRVs* transmitted vertically between all sheep.

When these experiments were started there were only two nucleotide sequences available in the literature for JSRV: i) the complete nucleotide sequence of a South African strain of JSRV (York *et al*, 1992; ii) a portion of *gag* from a Peruvian strain of JSRV (Hecht *et al*, 1994).

The effort of searching possible differences between exogenous and endogenous viruses was therefore initially concentrated on a portion of the *gag* gene where major information was available. Thus, RT-PCR amplification of a portion of JSRV-*gag* from tissues collected from SPA-affected sheep and unaffected controls was initially performed. Differentiation between exogenous and endogenous RNA species was then evaluated by sequencing and/or restriction enzyme digestion of PCR products

obtained from viral pellets and tumour of SPA-affected sheep and tissues of unaffected controls.

4.2 Materials and methods.

4.2.1 Sources and preparation of samples.

Samples were derived from the SPA-affected and unaffected sheep as described in 2.1.

Lung fluid samples (2.5-5 ml; n= 16) were collected during clinical examination of SPA-affected sheep; two of the samples were collected from SPA-affected sheep in Spain. Lung fluids were filtered, clarified and ultracentrifuged as described in 3.2.3 except that the resultant pellet was resuspended in 500 µl of solution D and stored at -20°C until use. Leukocytes were obtained from blood samples (n=10) in which the red cells had been lysed. Approximately 10^7 cells for each sample were resuspended in 500 µl of solution D and stored at -20°C.

Lung tumour (n=5), normal lung (n=5), mediastinal lymph nodes (n=8), thymus (n=5), bone marrow (n=6), spleen (n=5) and kidney (n=6) of both SPA-affected and unaffected sheep were collected at post-mortem examination. Dog lung tissues were obtained from the Veterinary Pathology Department of the Royal (Dick) School of Veterinary Studies (Edinburgh) and were treated as the sheep tissues and used as negative controls. An equine dermis cell line and primary foetal lamb lung and kidney cells (10^7 cells for each sample) were obtained from the tissue culture service at Moredun Research Institute and employed as further controls in this study. Cells were lysed in 500 µl of solution D and stored at -20°C until examined.

4.2.2 RNA extraction.

Total RNA from ultraspeed pellets of lung fluid (2.5 - 5.0 ml), sheep tissues and dog lung tissues, leukocytes (10^7), equine dermis cell line, foetal lamb lung and kidney

cells (10^7 cells) was extracted as described in 2.3.18. In addition, RNA was extracted from one goat nasal tumour viral pellet prepared as in 3.2.3.

Each RNA sample was treated with DNase I (Sigma) as described in 2.3.18 to avoid genomic DNA contamination. As a further control, some of the samples processed at the beginning of the study were additionally treated with RNase A (Sigma). RNase A was added to the RNA preparation at a final concentration of 50 $\mu\text{g/ml}$ and the samples were incubated for an hour at 37°C .

The quality of the RNA extracted was assessed by running an aliquot of the sample (2 μl) onto a 1% denaturing formaldehyde/agarose gel as described in 2.3.20 and the RNA concentration was estimated by the use of a UV spectrophotometer (see 2.3.4).

4.2.3 Genomic DNA extraction.

Genomic DNA was obtained from lung tumour and kidney collected from four SPA-affected sheep and two unaffected controls as described in 2.3.21.

4.2.4 cDNA synthesis.

Synthesis of cDNA was carried out in 25 μl reaction volumes as follows: total RNA (2 μg from tissues, leukocytes and cell cultures samples or total RNA obtained from ultraspeed pellets of 2.5-5 ml of lung fluid) was diluted in 13.5 μl DEPC-treated distilled water. RNA was denatured at 65°C for 10 min and immediately cooled on ice. A 1 μl (40 U) of rRNAsin (Promega), 2 μl of 25 mM dNTPs, 2.5 μl 100mM DTT, 2.5 μl (62.5 A_{260} U/ml [Boehringer]) random hexamers, 2.5 μl 10X reaction buffer (500mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2) and 1 μl of StrataScript™ reverse transcriptase (50 U, Stratagene) were added. The addition of the reverse transcriptase was omitted in control samples to check for DNA contamination of the RNA preparations. The reaction mixture was incubated at 37°C for 1 h, and then the enzyme was inactivated by incubation at 94°C for 3 min.

4.2.5 JSRV-*gag* PCR.

Amplification of the resultant cDNA was carried out by PCR. Primers were based upon the published sequences of JSRV (York *et al*, 1992) and pCA1 (Hecht *et al*, 1994). Primers P1 and P2 spanned a 229 bp region internal to the *gag* gene ([position 1598-1826 of JSRV] (sense -P1-: 5'-GCTGCTTTRAGACCTTATCGAAA-3'; antisense-P2-: 5'-ATACTGCAGCYCGATGGCCAG-3')). PCR was optimised using plasmid pJS382 (courtesy of Dr. G. Querat) containing part of the JSRV-*gag* gene (nt 953-3029) as template.

The optimised protocol was as follows: 3 µl of cDNA or 500 ng of genomic DNA was added to 47 µl of 1 x PCR buffer (1.25U *Taq* polymerase [Boehringer], 2.75 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 200 µM of each dNTP, 6.25 pmol of each primer). PCR cycles employed were 94°C for 1 min and 35X (94°C for 45 sec, 57°C for 1 min, 72°C for 1 min), with a final extension of 72°C for 2 min.

Amplified products were detected by agarose gel electrophoresis as described in 2.3.2.

'Reconstruction' experiments were performed to establish the sensitivity of the test employing plasmid pJS382. Serial dilutions of pJS382 were amplified with or without the addition of 500 ng of normal sheep kidney genomic DNA.

4.2.6 Southern blotting.

Specificity of the amplified product was determined by Southern hybridisation with a ³²P end labelled oligonucleotide probe. The probe (PA= 5'GGCTTGCTGCTG AAAAGTACT3'; nt 1726-1747 of JSRV) was designed to anneal to an internal region of the amplified product. Southern blotting was performed as described in 2.3.16 with a hybridisation temperature of 42°C and washings at 50°C with 0.1 x SSC.

4.2.7 Cloning of gag-PCR products.

The products of five independent PCR-*gag* reactions using kidney cDNA from an unaffected sheep were pooled before cloning in pGEM-T (Promega) as described in 2.3.10 - 2.3.13. Seven clones were selected and sequenced using the T7-sequencing kit (Pharmacia) as described in 2.3.15.

Plasmid DNA from one of the clones (*enJSRV-gag1*) was also used as source of endogenous DNA in 'reconstruction' experiments explained in the results session.

4.2.8 Restriction enzyme digestion.

A 25 µl aliquot of the PCR-*gag* product were digested with 13 U of *ScaI* (Boehringer) employing the appropriate incubation buffer supplied by the manufacturers (PCR buffer was ignored) at 37°C for 2-4 hours. The digestion products were analysed alongside the corresponding mock-digested PCR products, by electrophoresis in a 2% agarose gel stained with ethidium bromide.

4.3 Results.

4.3.1 Detection of *enJSRVs-gag* transcripts.

The analysis of cDNA samples from both tissues collected from SPA-affected animals and unaffected control sheep was performed by RT-PCR-*gag*.

A product of the expected size (229 bp) was detected in all 65 cDNA tissues tested. cDNA from primary cultures of foetal lamb lung and kidney cells produced the same visible amplicon whereas dog lung tissues and an equine dermis cell line were consistently negative. All the samples were treated with DNase to exclude DNA contamination. In addition, all the samples were tested in parallel omitting reverse transcriptase from the reaction mix; no signal was obtained from any of these samples. Neither were signals obtained from the aliquots of the samples which were

treated with RNase (Fig 4.1a). The specificity of the amplimers was confirmed by Southern hybridisation with an oligonucleotide probe that hybridised to an internal portion of the PCR product (Fig. 4.1b).

Lung fluid of SPA-affected sheep contains JSRV particles (see chapter 3), therefore it may be assumed that the RNA detected in these materials is of exogenous origin while the RNA detected from tissues of unaffected control animals is transcribed from endogenous viruses. The results of these experiments therefore demonstrated that transcripts of endogenous origin are transcriptionally active in a variety of sheep tissues.

4.3.2 Differentiation between endogenous and exogenous transcripts.

The working hypothesis of this experiment was that JSRV is an horizontally transmitted virus and is distinguished from its endogenous counterparts.

To identify possible differences between endogenous and exogenous RNA, the RT-PCR products obtained from the kidney of an unaffected control animal were cloned and sequenced. Seven clones were sequenced and four different sequences (*enJSRV gag1-4*) were obtained. The four *enJSRV* sequences were compared with each other and with the published sequences, JSRV (York *et al*, 1992) (South African isolate) and pCA1 (Hecht *et al*, 1994) (Peruvian isolate) by computer analysis using the GCG “pileup” program (Genetic Computer Group, 1994). The South African and the Peruvian isolate were 90% similar in the stretch of sequence considered. The *enJSRV* clones were highly homologous to each other (95 - 99% homology), 95-98% homologous to the South African isolate and 85-88% to the Peruvian isolate. Comparison of the restriction map of the six sequences revealed a *ScaI* restriction site in JSRV and pCA1 that was absent in all the *enJSRV* clones (Fig 4.2).

To confirm this observation, RT-PCR products from 16 lung fluids, 5 tumours and 44 non-tumour tissues were digested with *ScaI*. Successful digestion, indicated by the presence of two smaller fragments of 131 and 98 bp, was observed with all 16 lung fluid samples, 5 out of 5 tumours and faintly in 3 out of 5 mediastinal lymph

nodes from SPA-affected sheep (Fig 4.3). No *ScaI* sensitive product was observed with any other tissue of either healthy controls or SPA-affected sheep. The results are summarised in Table 4.1.

These findings established for the first time a molecular marker for the exogenous form of JSRV.

The ENTV sample was successfully amplified by PCR-*gag* but it was not digested by *ScaI* treatment.

4.3.3 Lack of detectable JSRV at DNA level.

PCR products obtained by direct amplification of tumour and kidney genomic DNA with primers P1 and P2 followed by *ScaI* digestion did not show any cut product by electrophoresis in an agarose gel stained with ethidium bromide. The sensitivity of the PCR-*gag/ScaI* digestion to detect exogenous sequences against a background of endogenous sequences was assessed by 'reconstruction' experiments. In the first series of experiments the capacity of *gag*-PCR/*ScaI* to detect exogenous sequences (e.g. plasmid JS382 DNA) with or without a background of 500 ng of genomic DNA was compared. The two characteristic bands of 131 and 98 bp were still visible when $10 - 10^2$ copies of pJS382 were amplified by PCR-*gag* followed by *ScaI* digestion. However, no visible product was visible when 10^5 copies of pJS382 were amplified along with 500 ng of kidney DNA (Fig. 4.4). In a second set of experiments a fixed quantity (10^2 copies) of pJS382 were amplified with serial dilution ($10^2 - 10^7$) of *enJSRV-gag1*. After *ScaI* digestion, exogenous sequences were detected only when the exogenous/endogenous ratio was 1:10 but not when it was 1:100.

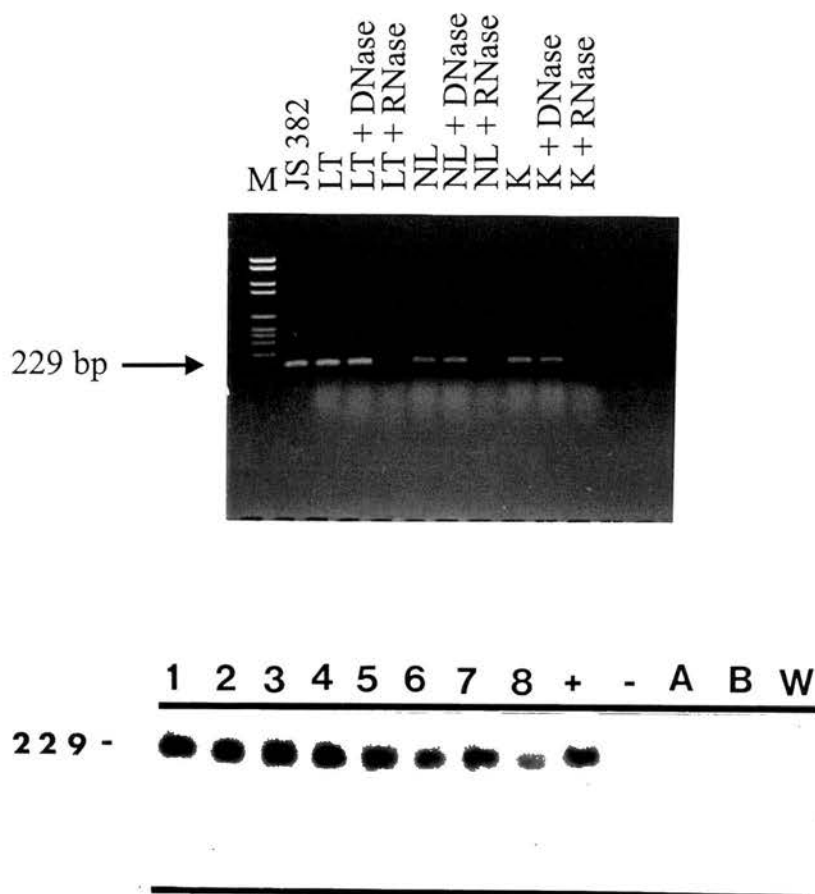


Fig. 4.1: **JSRV-*gag* PCR.** RT-PCR *gag* showing the amplification of a product of the expected size from tissues of both SPA-affected and unaffected control sheep (top). JS382, plasmid containing the target sequence used as positive control; LT, lung tumour from a SPA-affected sheep; NL, normal lung and K, kidney from an unaffected control sheep. Treatment of the RNA sample with DNase or RNase is indicated.

The bottom panel shows a Southern blot of JSRV-*gag* RT-PCR products employing a radiolabelled oligonucleotide probe (probe A). Lanes 1-4, lung tumours from SPA-affected sheep; lanes 5-8, tissues from unaffected control sheep. Lane 5, lung; lane 6, spleen; lane 7, kidney; lane 8, mediastinal lymph node. +, positive control is plasmid JS382; lane A, equine dermis cell line; lane B, dog lung; W, distilled water.

[illegible]

Fig. 4.2: Comparison of *env/SRV gag* sequences and exogenous pCA1(Hecht *et al.*, 1994) with JSRV (York *et al.*, 1992). Indicated are base differences with JSRV. Primers used are underlined while in bold is indicated the exogenous specific *Scal* site.

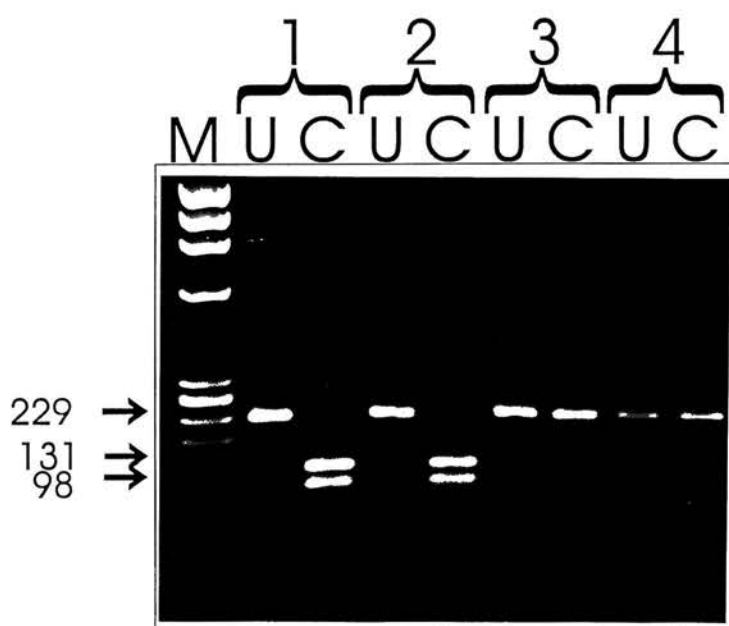


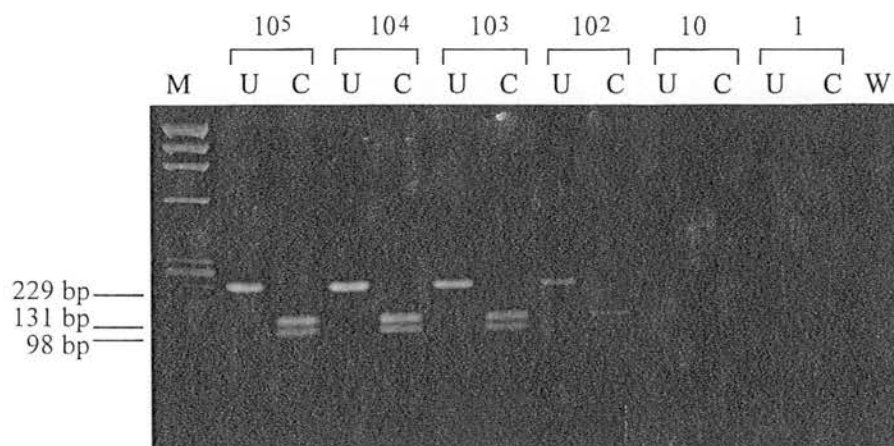
Fig. 4.3: Typical results of RT-PCR, followed by *ScaI* digestion, of total RNA from SPA-affected sheep tissues. Sample 1, lung fluid; sample 2, lung tumour; sample 3, mediastinal lymph nodes; sample 4, kidney (samples 1 to 4 were collected from the same animal. U= mock digested PCR products. C= PCR product digested with *ScaI*. M= molecular weight marker IX (Boehringer).

Tab. 4.1 : Tissues examined by RT-PCR *gag* followed by *ScaI* digestion in SPA-affected and unaffected control sheep.

| Tissues | SPA+ ^a | | SPA- ^b | |
|-------------------------|--------------------------|--|--------------------------|--|
| | PCR (tested/positive) | <i>ScaI</i> digestion (tested/sensitive) | PCR (tested/positive) | <i>ScaI</i> digestion (tested/sensitive) |
| Lung fluid | 16/16 | 16/16 | - | - |
| Tumour/Lung | 5/5 | 5/5 | 4/4 | 4/0 |
| Kidney | 3/3 | 3/0 | 3/3 | 3/0 |
| Thymus | 2/2 | 2/0 | 3/3 | 3/0 |
| Bone marrow | 3/3 | 3/0 | 3/3 | 3/0 |
| Spleen | 2/2 | 2/0 | 3/3 | 3/0 |
| Mediastinal lymph nodes | 5/5 | 5/3 | 3/3 | 3/0 |
| Leukocytes | 5/5 | 5/0 | 5/5 | 5/0 |
| Total | 41/41 | 41/24 | 24/24 | 24/0 |

^a SPA affected sheep.

^b Unaffected control sheep.



+ 500 ng normal sheep
genomic DNA

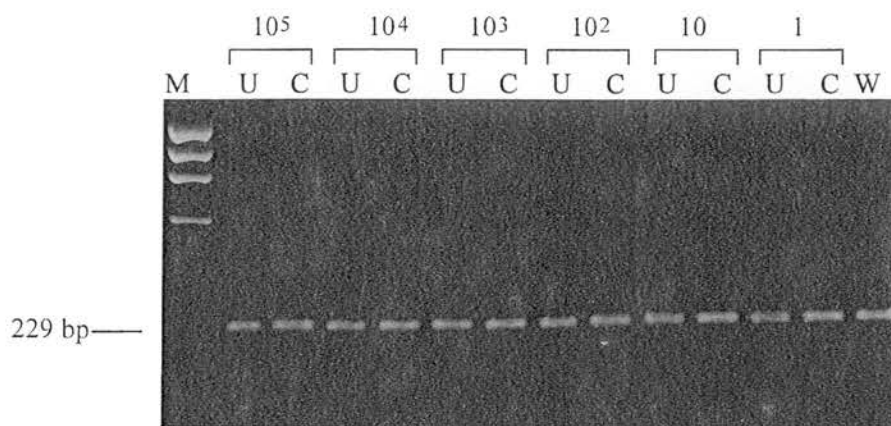


Fig. 4.4: **Sensitivity of JSRV-*gag* PCR followed by *ScaI* digestion.** Serial dilutions of plasmid JS382 containing the JSRV *gag* template were amplified with (bottom) or without (top) a background of 500 ng of sheep genomic DNA collected from an unaffected control sheep. The estimated number of plasmid copies is indicated at the top of each gel. Lanes: U, mock-digested PCR product; C, PCR product digested with *ScaI*; M, molecular weight marker IX (Boehringer).

4.4 Discussion.

In this chapter, compelling lines of evidence have been presented which collectively demonstrate a specific association between the exogenous form of JSRV and SPA.

It was shown that the exogenous form of JSRV was distinct from endogenous JSRV transcripts as identified by the presence of a *ScaI* restriction site in *gag* (nt position 1729 [nucleotide position referred to York *et al*, 1992]). Furthermore, this site was conserved in strains of exogenous JSRV from geographically dispersed countries, confirming and extending data linking these viruses to each other and to SPA (Herring *et al*, 1983; Sharp and Herring, 1983; Payne *et al*, 1986; Rosadio 1988b; He *et al*, 1992).

Exogenous JSRV RNA was found only in tumour, lung fluid and draining lymph nodes of SPA-affected sheep, which is in agreement with the immunological data shown in chapter 3, which concluded that epithelial tumour cells are major sites of replication for JSRV. Lung fluid and tumour from SPA-affected sheep are the only materials that have been used successfully to reproduce the tumour experimentally in sheep and goats (Martin *et al*, 1976; DeMartini *et al*, 1987; Sharp *et al*, 1983; Sharp *et al*, 1986; Verwoerd *et al*, 1980). These findings, therefore, point to exogenous JSRV as a strong candidate for the aetiological agent of SPA.

The failure to detect *ScaI* sensitive sequences at the DNA level is probably due to the high proportion of endogenous template compared to exogenous, resulting in a small proportion of *ScaI* sensitive product. Theoretically, there may be 20 endogenous copies compared to possibly only one exogenous copy per neoplastic cell. Moreover, neoplastic cells are approximately only 40-50% of the total number of cells present in the neoplastic tissue, therefore increasing the ratio of endogenous to exogenous sequences. The results obtained with the 'reconstruction' experiments seem to support this hypothesis. However, until the exogenous JSRV provirus is detected in tumour DNA and not in non-tumour DNA from the same SPA-affected sheep, it will not be possible to rule out that the exogenous virus derives from an endogenous virus

which is transcriptionally silent in non-tumour tissues and is activated either as a pre-neoplastic or post-neoplastic event.

The presence of transcriptionally active *enJSRVs* raises questions about their potential role in SPA. As mentioned in 3.4 endogenous retroviruses have been demonstrated in mice, chicken and cats to be involved in recombination events with exogenous retroviruses and between themselves leading to the generation of oncogenic retroviruses or viruses with a broader host range (Weiss *et al*, 1973; Hartley *et al*, 1977; Stoye *et al*, 1991; Golovkina *et al*, 1994; McDougall *et al*, 1994; Bai *et al*, 1995). The possibility of recombination events between the exogenous form of JSRV and its endogenous homologues cannot be ruled out.

An interesting finding outlined in this chapter, was the detection of exogenous JSRV RNA in 3 out of 5 mediastinal lymph nodes of SPA-affected sheep. Previous immunological studies (chapter 3) detected JSRV particles in only 1 out of 12 regional lymph nodes of SPA-affected sheep. The detection of the exogenous JSRV in the majority of regional lymph nodes by RT-PCR may reflect the presence of metastases, although these were not detected by histological examination (performed by Dr. M. De las Heras, data not shown), or may reflect the presence of the virus in the lymph drained by the regional lymph nodes at levels below the detection limit of the immunological techniques. Due to the lack of data on the immune response to JSRV we cannot determine whether the presence of JSRV in the lymph nodes is related to an active immune response towards the virus.

To date, JSRV has not been isolated *in vitro* and therefore classical approaches to demonstrate the aetiology of SPA have been impossible. However, the results reported in this chapter show that exogenous JSRV RNA is distinct from its endogenous JSRV-related transcripts and is consistently and exclusively demonstrated in lung tumours of SPA-sheep. This association would support the hypothesis that JSRV is an horizontally transmitted virus directly involved in the aetiology of SPA.

Interestingly ENTV has been amplified by PCR-*gag* underlining the similarity with JSRV. However, the nasal tumour virus lacks apparently the *ScaI* site which could indicate that these two viruses although related have to be considered distinct.

Chapter 5.

Detection of JSRV Provirus in Tumour Genomic DNA.

5.1 Introduction.

In chapter 3 the presence of JSRV particles and antigens in the epithelial tumour cells of the SPA-affected sheep was described. In chapter 4, JSRV RNA was differentiated from its endogenous counterparts by the presence of a unique *ScaI* site in a fragment of *gag* and was detected only in the respiratory tissues of SPA-affected sheep. In both cases the detection of JSRV was restricted to sheep affected by SPA and never in healthy control animals.

To demonstrate firmly that the exogenous JSRV, present in tumour and lung fluid of SPA-affected sheep, is a horizontally transmitted virus and does not derive from a reactivated *enJSRV* it is necessary to detect JSRV in tumour DNA and exclude its presence in non-tumour (e.g. kidney) DNA collected from the same SPA sheep.

The initial approach was to detect the exogenous virus by a selective PCR amplification, based on the assumption that if one of the primers was designed on the exogenous-specific *ScaI* restriction site the reaction will successfully amplify only the exogenous JSRV. Specific amplification of sequences differing by as little as a single nucleotide, known as ‘allele-specific PCR’ (Ugozzoli and Wallace, 1991) or ‘mismatch amplification mutation assay (MAMA)’ (Cha *et al*, 1992), has been applied successfully on a number of occasions for polymorphism analysis and for the detection of rare mutations (Newton *et al*, 1989; Wu *et al*, 1989; Ehlen and Dubeau, 1989; Nichols *et al*, 1989; Ferrie *et al*, 1992).

Primer-template complexes containing mismatches have lower melting temperatures than perfectly matched complexes and, consequently, a PCR performed at the appropriate annealing temperature will promote amplification only of the stably annealed primer-template. While single internal mismatches have no significant effect on PCR product yield, those at the 3’ end of the primers enhance specificity since DNA polymerases extend mismatches much less efficiently than correct matches (Petruska *et al*, 1988; Perrino *et al*, 1989; Creighton *et al*, 1992) and *Taq*

DNA polymerase lacks a 3' → 5' exonuclease activity (Tindall *et al*, 1988; Eckert *et al*, 1990).

A second approach to detect exogenous JSRV at the DNA level has been the result of an extensive collaboration with Dr. DeMartini's Laboratory at the Department of Pathology of the College of Veterinary Medicine and Biomedical Sciences at Colorado State University, who made available an LTR sequence obtained by PCR amplification of one of the *enJSRVs*. The endogenous LTR sequences appeared to have major differences in the U3 region compared to the only exogenous LTR sequence published by York *et al* (1992). After a preliminary confirmation of this finding, it was reasoned that it would be possible to design an exogenous-specific primer in the U3 region of JSRV and amplify a portion of the proviral JSRV comprising the LTR and *gag* (downstream the exogenous-specific *ScaI* site). In that way, detection of exogenous JSRV would be confirmed by the presence of the extensively characterised *ScaI* site.

5.2 Materials and Methods.

5.2.1 Genomic DNA extraction.

Genomic DNA was extracted from lung tumour and kidney samples of four SPA-affected animals and from lung and kidneys of three unaffected controls as described in 2.3.21. DNA was also isolated from primary cultures of normal sheep skin fibroblasts for the analysis of the *enJSRVs* LTRs (see below).

Plasmids JS382 (York *et al*, 1992) and *enJSRV-gag1* containing, respectively, the exogenous and endogenous *gag* template were used as DNA sources for the reconstruction experiments of the allele-specific PCR.

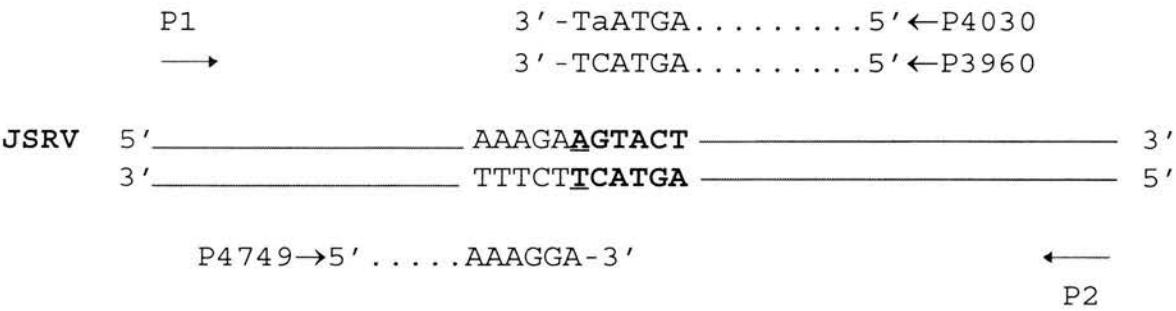
5.2.2 Allele-specific PCR (AS-PCR).

Three sets of reactions were performed (Fig 5.1). In the first reaction (#1), primer P1 (sense primer for the PCR-*gag* described in chapter 4) was coupled with primer P3960 (antisense, position 1747-1726; 5'-GGCTTGCTGCTGGAAAAGTACT-3'). The last nucleotide of P3960 (T) is complementary to the unique 'A' of the *ScaI* site of JSRV (Fig. 5.1[A]) and creates a T:G mismatch with the (known) sequences of *enJSRV* (Fig. 5.1[B]). In the second reaction (#2), primer P1 was coupled with primer P4030 (antisense, position 1747-1726; 5'-GGCTTGCTGCTGGAAAAGTAAT-3'). Primer P4030 creates a T:G mismatch with the endogenous sequences the same as P3960 (Fig. 5.1[B]) but has an additional arbitrary mismatch at the penultimate base (an 'A' instead of a 'C') which further increases the instability of the primer. In the third reaction (#3), primer P2 (antisense primer for the PCR-*gag* described in Chapter 4) was coupled with primer P4749 (sense, position 1700-1726; 5'-GCATTACAAGGAAAAAGCATAAAAGGA-3').

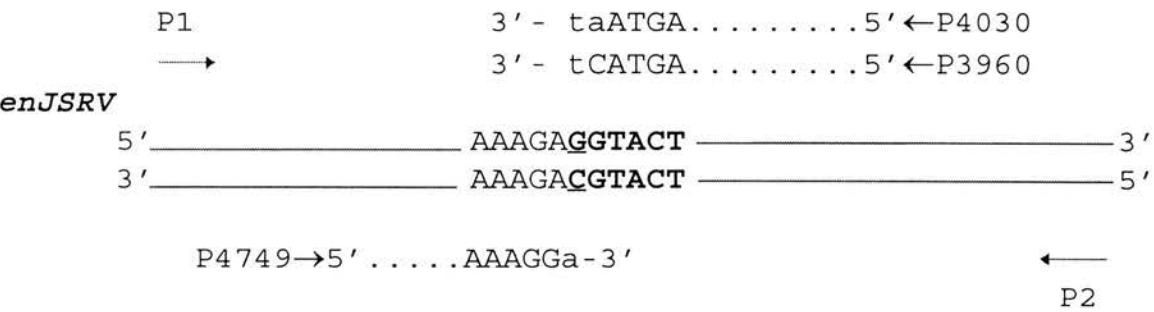
Templates in the PCR reactions were used either as 10 fold dilutions (from 10^6 to 10 copies) of plasmid JS382 and *enJSRV-gag1* in the reconstruction experiments or as matched tumour and kidney genomic DNA samples as above (5.2.1). One hundred and 500 ng of each sample were used in each reaction.

With the exception of *Taq* DNA polymerase [Boehringer] (1.25 U per reaction) and the PCR buffer (2.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl) the other parameters were varied and optimised to achieve the best specificity of the reaction. The concentration of dNTPs varied between 50 and 200 μ M and the primers concentration between 0.2 pmol and 6.25 pmol. The addition of DMSO to the reaction varied from 0 to 10%. Other variables were the number of cycles (25, 30, 35) used and the annealing temperature (57°C to 65°C). Other parameters in the cycles were identical to that of the PCR-*gag*.

A)



B)



- Reaction #1= P1 + P3960
- Reaction #2= P2 + P4030
- Reaction #3= P2 + P4749

Fig. 5.1: **Allele-specific PCR strategy.** The exogenous JSRV target is schematically represented in (A) while (B) represents the endogenous one. The *ScaI* site is represented in bold. Note that the 3' end of P4030, P3960 and p4749 is complementary to JSRV but not to *enJSRV* (mismatches of the primers are indicated in lower case). The second last base of primer P4030 has an arbitrary mismatch with both templates.

5.2.3 Amplification and sequencing of endogenous LTRs.

One hundred ng of genomic DNA isolated from sheep skin fibroblasts was used for PCR amplification of endogenous LTRs (PCR-LTR). Primers were designed at the 5' end of the U3 (sense - P3 - CTGCGGGGGACGAC, position 7179-7193 [York *et al*, 1992]) and at the 3' end of U5 (antisense - P4 - CTGCCGCGACCAGCA, position 111-125). The buffer employed was 10 mM Tris pH 8.9, 50 mM KCl, 2 mM MgCl₂ with 200 µM each dNTP, 100 ng primers and 1 U of *Taq* (Boehringer). PCR cycles employed were an initial step of 94°C for 2 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min with a final extension of 72°C for 2.5 min. The products of three independent PCR reactions were pooled and then cloned in the TA cloning system (Invitrogen) following the manufacturer's instructions. Three resultant clones were sequenced by the dideoxy chain termination method employing a Li-Cor automated sequencer. The nucleotide sequence of the PCR clones of the LTRs are deposited in GenBank under accession numbers Z66531, Z66532, and Z66533 (*enJSRV LTR* 1-3).

The work described in this paragraph was performed by Dr. R.G. Dalziel (Veterinary Pathology Department, Royal (Dick) School of Veterinary Studies, University of Edinburgh).

5.2.4 Hemi-nested LTR-*gag* PCR.

In the first round of amplification, 250-500 ng of tumour and kidney high molecular weight DNA was amplified by PCR employing the Expand™ Long Template PCR system (Boehringer) which uses a mixture of *Taq* and *Pwo* DNA polymerases. The sense primer (-P5- TGGGAGCTCTTTGGCAAAAGCC - nucleotide positions 7210-7224) was designed on the basis of nucleotide sequence differences between the endogenous LTR sequences and the JSRV sequence. Primer P2 (antisense primer of the PCR-*gag* described in chapter 4) was employed as antisense primer. Reactions were carried out in a 50 µl volume containing 50 mM Tris-HCl, pH 9.2, 160 mM

(NH₄)₂SO₄, 2.25 mM MgCl₂, 2% (v/v) DMSO, 0.1% (v/v) Tween® 20, 500 µM each dNTP, 300 nM each primer, template DNA and 2.625 U of enzyme mix. Cycles were as follows: 1 cycle at 92°C for 2 min, 30 cycles at 92°C for 10 sec, 61°C for 30 sec and 68°C for 90 sec (plus a progressive increment of 20 seconds from cycle 11 onwards), and a final extension of 68 °C for 7 min.

One µl of product of the first round of amplification was used in the second round of PCR which was the same as the PCR-*gag* described in chapter 4. Half the product of the second stage PCR was digested with *ScaI* as described above. The strategy of the hemi-nested LTR-*gag* PCR is illustrated in Fig 5.2.

Products of the first and the second rounds of heminested PCR were visualised by electrophoresis in 1 and 2% agarose gels respectively.

5.3 Results.

5.3.1 Allele-specific PCR.

An allele-specific PCR approach was attempted in order to successfully amplify exogenous JSRV at DNA level. To demonstrate that JSRV has an exogenous origin only and it is not a reactivated endogenous virus it is necessary to detect JSRV at DNA level in tumour but not in non-tumour (e.g. kidney) DNA collected from the same sheep. The efficiency of an allele-specific PCR depends largely on the position and nature of the primer:template duplex. Three primers were designed with their 5' end complementary to the putative exogenous-specific *ScaI* site in *gag* and used in three different sets of reactions (Fig. 5.1) coupled to P1 or P2 described in Chapter 4. Under stringent conditions the AS-PCR should have been able to detect only the exogenous provirus (e.g. with the unique *ScaI* site). An endogenous (*enJSRV-gag1*) and an exogenous (pJS382) clone were used in reconstruction experiments to optimise the AS-PCR conditions.

Reaction #1 did not distinguish between the exogenous and the endogenous template regardless of the different conditions used. In reconstruction experiments reactions

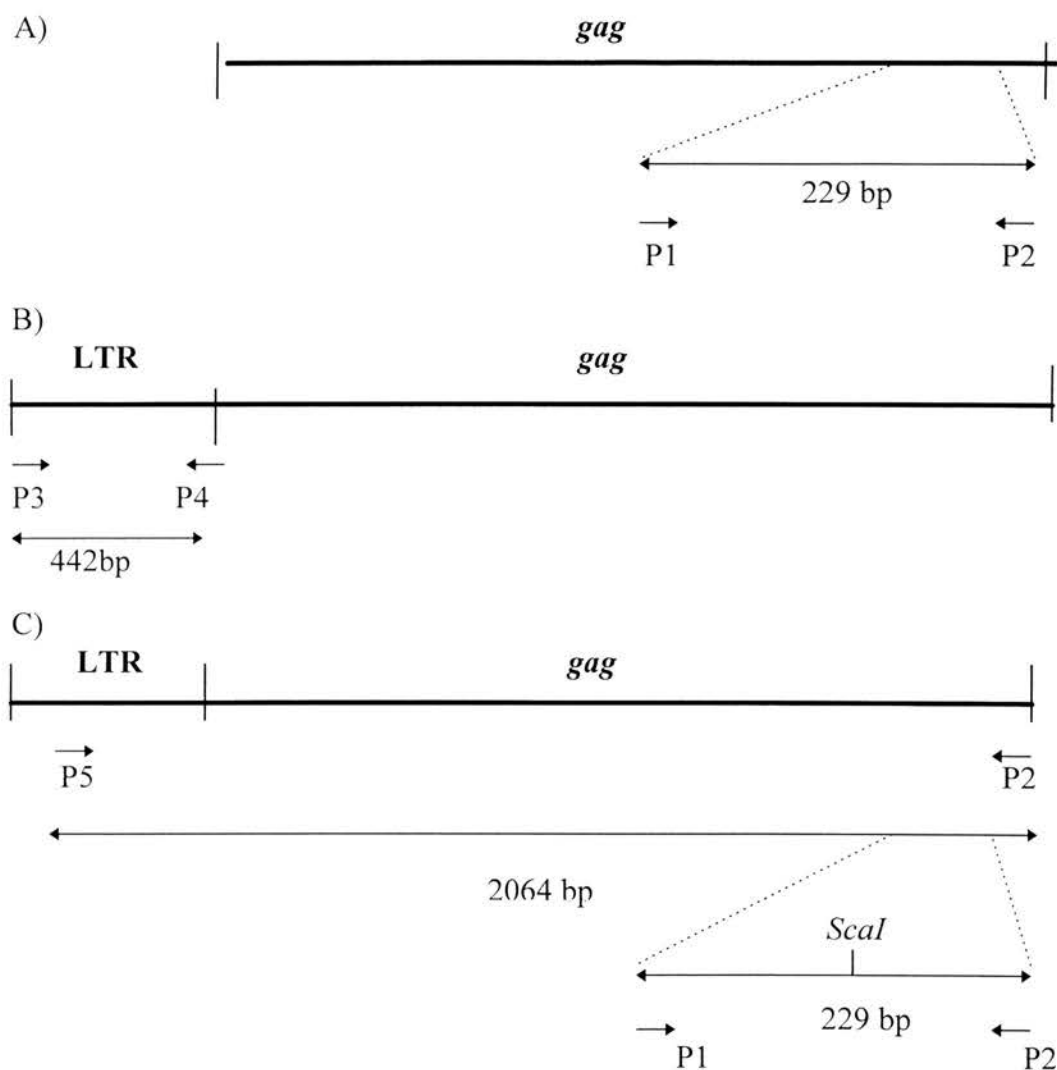


Fig. 5.2: **PCR strategy and localisation of primers.** (A) RT-PCR *gag* employing primers P1 and P2 amplified a product of 229 bp. (B) PCR for the amplification of endogenous LTR employed primers P3 and P4 and generated products of 442/444 bp. (C) Hemi-nested LTR-*gag* PCR. The first round of amplification used primer P5 (internal to the LTR) and *gag* primer P2. 1 μ l of this reaction was used in the second round of amplification which used primers P1 and P2.

#2 and 3 were able to preferentially amplify the exogenous plasmid DNA. The best conditions found for the two reactions were the following: 10 mM-Tris-HCl, 50 mM KCl, 2.75 mM MgCl₂ with 50 µM each dNTP, 6% DMSO, 6.25 pmol of each primer and 1.25 U of *Taq* polymerase (Boehringer) in a 50 µl reaction. Each sample subjected to 94 °C for 1 min followed by 35 cycles of 94°C for 45 sec, 63°C for 1 min and 72°C for 1 min with a final extension of 72°C for 2 min.

Both reaction #2 and #3 were able to amplify 10⁵ copies of endogenous clones but not 10⁴ while they amplified 10³ copies of exogenous plasmid pJS382 (Fig 5.3a).

The AS-PCR was then applied to genomic DNA. Neither lung tumour nor kidney genomic DNA were successfully amplified with either reaction #2 or #3 at the above specified conditions, implying that the PCR reactions were not sensitive enough to detect the exogenous virus when genomic DNA was present as background (5.3b).

5.3.2 Amplification and sequencing of endogenous LTRs.

PCR products of approximately 450 bp were obtained from the amplification of DNA from cultured sheep primary skin fibroblast cultures employing primers P3 (end of U3) and P4 (end of U5). Products were cloned as described in materials and methods and three clones were completely sequenced (Fig.5.4).

The *enJSRV* LTRs appeared to be longer (442 to 445 bp) than the published exogenous LTR (397 bp) which showed two large deletions at positions 198 and 261 of 30 and 16 base pairs respectively (nucleotide positions as in Fig.5.4). The nucleotide sequence of the three *enJSRV* LTR were 83 to 90% identical and were 69 to 76% homologous to the JSRV LTR sequence (York *et al*, 1992). Major differences between *enJSRVs* and JSRV LTR were found in the U3 and in particular in the 5' portion of the U3 where the homology was around 50%. The R regions of the *enJSRVs* clones were identical to R of JSRV while the U5 were 84 to 91% homologous to JSRV.

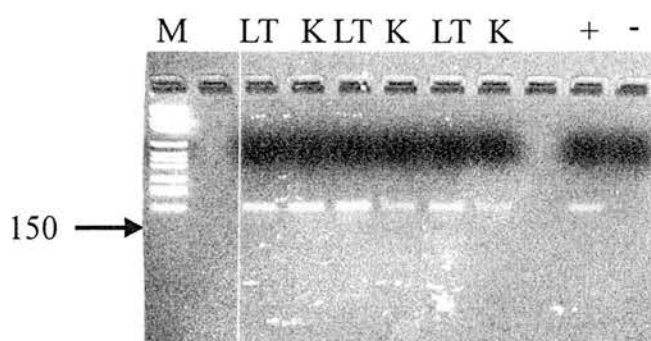
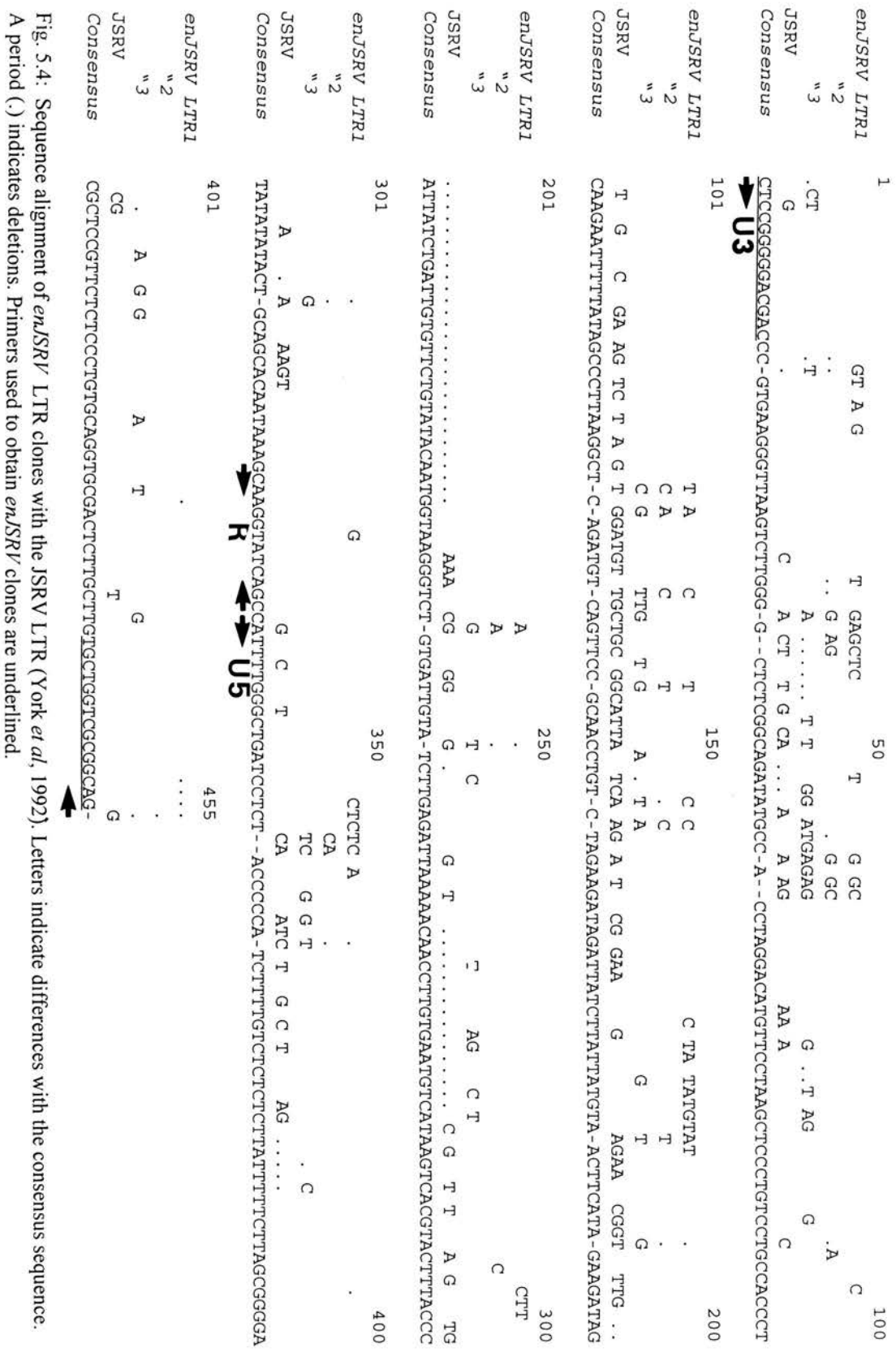


Fig. 5.3: Example of the results obtained with the AS-PCR (reaction #2). In the top panel are shown reconstruction experiments with endogenous (*enJSRV*) and exogenous (*JS382*) clones. The estimated number of copies is indicated in each lane. M= DNA molecular marker VI (Boehringer); W= distilled water. In the bottom panel are shown experiments with genomic DNA from lung tumour (LT) and kidney (K) of three SPA-affected sheep; += plasmid *JS382* used as positive control; -= negative control (water).



5.3.3 Detection of JSRV provirus in tumours of SPA-affected sheep.

As it was shown in chapter 4, PCR products obtained by direct amplification of tumour genomic DNA with primers P1 and P2 followed by *ScaI* digestion did not show any cut product by electrophoresis in an agarose gel stained with ethidium bromide. The failure to detect *ScaI* sensitive sequences at DNA level may be due to the high proportion of endogenous template compared to exogenous, resulting in a small proportion of *ScaI* sensitive product.

To preferentially amplify exogenous proviral DNA sequences we designed a hemi-nested PCR. The first round of amplification used a sense primer (P5) based on an area of sequence divergence between endogenous LTR clones and the exogenous LTR sequence (JSRV) and the antisense primer (P2) of the RT-PCR *gag*. A product of about 2 kb was visible in an ethidium bromide agarose gel from 3 out of 4 SPA tumour DNAs whilst no signal was obtained in 4 out of 4 kidney DNAs of the same animals or from lung and kidney of three unaffected control animals. A second round of amplification, using primers of the RT-PCR (*gag*) followed by *ScaI* digestion, enabled the detection of *ScaI* sensitive sequences in all four tumours examined, whilst only spurious bands were detected in non-tumour samples of SPA-affected or non-affected sheep (Fig. 5.5).

5.4 Discussion.

The aim of the studies conducted in this chapter was to prove that JSRV is an exogenous virus and does not derive from a reactivated *enJSRV*.

In chapter 4, a *ScaI* restriction site in *gag* was established as an exogenous-specific marker for JSRV RNA. Experiments therefore, were concentrated on finding this marker at the DNA level. The initial approach to utilise an allele-specific PCR was unsuccessful although it was proved theoretically possible. In reconstruction experiments, two of the three PCR reactions were able to preferentially amplify the exogenous clone more than one hundred times with respect to the endogenous one.

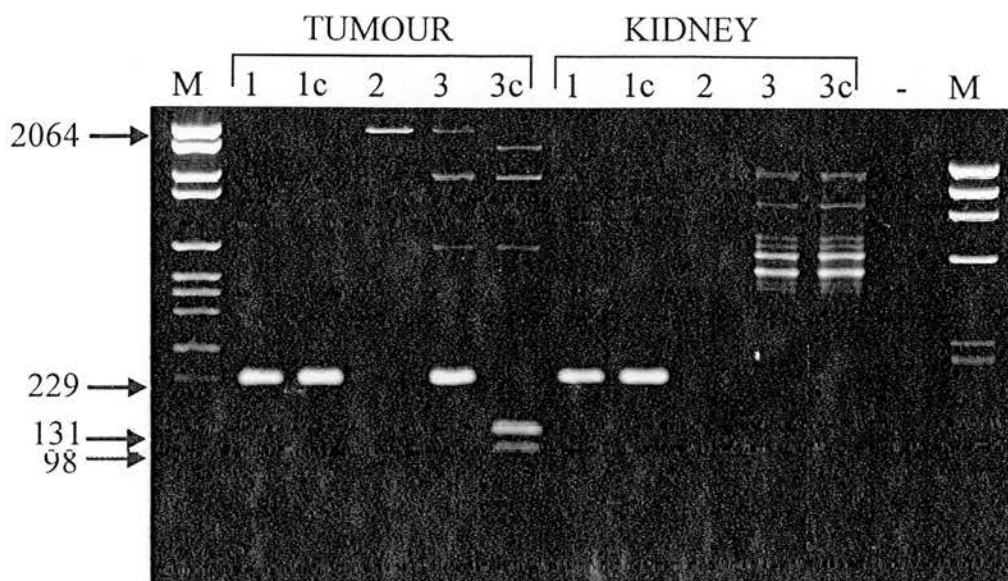


Fig. 5.5: **Detection of exogenous JSRV sequences in tumour and kidney DNA of the same SPA-affected sheep.** The gel is divided in two halves with the tumour sample on the left and the kidney sample on the right as indicated. Lanes 1: 500 ng lung tumour or kidney genomic DNA amplified directly by PCR-*gag* employing primers P1 and P2; lanes 1c: PCR product shown in lane 1 cut with *Scal*; lanes 2: first round of amplification of hemi-nested LTR-*gag* PCR employing primers P5 and P2; lanes 3: second round of amplification, employing primers P1 and P2, of 1 μ l of the PCR product shown in lanes 2; lanes 3c: PCR product of lanes 3 cut with *Scal*. The minus (-) represents the negative control. M= Boheringer molecular weight markers VI (left) and IX (right).

However, the two reactions were not sensitive enough to pick up the exogenous virus in tumour DNA with the endogenous viruses as background. More primers could have been designed and tried but the availability of endogenous LTR sequences, where major differences were found with respect to the LTR sequence characterised by York *et al* (1992), made possible a more straightforward approach based on a two-step PCR (hn-LTR-gag PCR).

One of the two primers, employed in the first step of the hn-LTR-gag, was designed in the JSRV U3 which theoretically should correctly anneal only to exogenous template. Indeed, PCR products were obtained only from tumour DNA and not from the matched kidney samples of SPA-affected sheep. To demonstrate the exogenous origin of the template, an aliquot of this reaction was re-amplified by PCR-gag followed by *ScaI* digestion. The product obtained in the second step was entirely sensitive to treatment with *ScaI*. JSRV was thus demonstrated to be an horizontally transmitted virus specifically associated with SPA.

Differences in the LTR sequences between JSRV and the three *enJSRVs* PCR-derived clones were mainly present in the U3. A putative NF-1 motif in exogenous JSRV LTR (nt 7220-7223), which overlaps the exogenous specific PCR primer P5, is altered/lost in the endogenous LTRs and two tandemly repeated sequences (position 7347 to 7365 and 7366 to 7384 in JSRV sequence [York *et al*, 1992]) appear to be lost by means of an insertion of 30 nucleotides in the endogenous U3. Differences in the nucleotide sequence in these regions could potentially diminish/alter the LTR function and thus reduce the pathogenicity of *enJSRVs* compared to the exogenous counterpart as demonstrated in other retroviral systems (Lenz and Haseltine 1983; DeGroseillers and Jolicoeur, 1984; Speck *et al*, 1990; Hallberg *et al*, 1991; Plumb *et al*, 1991; DeGroseillers *et al*, 1993; Morrison *et al*, 1995; reviewed by Fan, 1990 and 1994).

The results obtained in this chapter and in the preceding two strongly suggest an aetiological role for JSRV in the induction of the tumour.

It is theoretically possible that JSRV is an exogenous non-pathogenic virus which actively replicates in type II pneumocytes only after their transformation. In this

context JSRV would infect SPA-affected sheep only after type II pneumocytes were transformed by other causes. Such a scenario seems extremely unlikely due to the 100% detection of JSRV in SPA cases in unrelated geographical areas and by the lack of detection of JSRV in unaffected control animals.

Chapter 6.

**Jaagsiekte Retrovirus Establishes a Disseminated Infection
of the Lymphoid Tissues of Sheep Affected by Pulmonary
Adenomatosis.**

6.1 Introduction.

Results presented in chapter 4 and 5 have demonstrated that JSRV is an exogenous virus specifically associated with SPA and distinct from the transcriptionally active endogenous retroviral sequences present within the ovine genome (sheep endogenous retroviruses, *enJSRVs* [syn: *enJSRV*]). A *ScaI* restriction site in *gag* has been defined as a molecular marker for the exogenous JSRV which has been consistently demonstrated in tumour and in draining lymph nodes of a proportion of SPA-affected sheep. JSRV was not detected outside the respiratory tissues of SPA-affected sheep nor in any tissue of control sheep (see chapter 4). Nevertheless, co-amplification of endogenous and exogenous sequences could have masked the detection of low copies of exogenous JSRV due to the relatively high background formed by *enJSRVs*.

To address this question an exogenous JSRV-specific hemi-nested PCR which amplified a segment of the U3 region of JSRV was designed. The anatomical distribution of JSRV in tissues collected from sheep with naturally and experimentally-induced SPA was then investigated.

6.2. Materials and Methods.

6.2.1 Samples employed in this study.

Samples of heparinised venous blood, lung fluid, lung, lung tumour, mediastinal lymph nodes, pre-scapular lymph nodes, mesenteric lymph nodes, spleen, femoral bone marrow, thymus, kidney and skin were collected during the post-mortem examination or immediately before (blood and lung fluid) from seventeen naturally SPA-affected sheep, six experimentally infected lamb and fifteen age- and breed matched control animals. Most of the samples were those collected for the investigations outlined in Chapters 4 and 5.

Tissue samples were snap-frozen in liquid nitrogen and stored at -70°C. Lung fluid samples were clarified by centrifugation at 10,000 x g for 1 h at 4°C and then stored

at -70°C. Plasma (5 ml) was clarified by centrifugation at 10,000 x g for 1 h at 4°C. The supernatant was then centrifuged at 100,000 x g for 1 h at 4°C. The pellet was resuspended in 500 µl of solution D and stored at -20°C. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by centrifugation over Lymphoprep™ at 800 x g for 30 min; aliquots of 4x10⁶ cells were stored at -70°C until RNA and DNA was extracted. Total RNA was extracted from 50 µl aliquots of lung fluid, 200-500 mg of lung tumour or from the plasma samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method as described in 2.3.18. PolyA⁺ RNA was obtained from 100 mg of all other tissues and from 4 x 10⁶ PBMC using the Microfast Track kit (Invitrogen) as described in 2.3.19. Genomic DNA was extracted from 10-25 mg of tissues and from 4 x 10⁶ PBMC using the Qiagen Tissue Amp kit as described in 2.3.21.

6.2.2 Amplification and sequencing of JSRV LTR.

The 5' LTR of a UK strain of JSRV was amplified from 500 ng of lung tumour genomic DNA isolated from a natural case of SPA using primer P5 and P2. These, formed the first primer pair of a hemi-nested LTR-*gag* PCR used to amplify proviral JSRV as described in 5.2.4. Briefly, the forward primer (P5) is located almost at the 5' end of the U3 while the reverse primer anneals to *gag* (P2) so that all but the first 33 bp of the JSRV 5' LTR are amplified (Fig. 5.2). In Chapter 5, primer P5 was demonstrated to be specific for the exogenous form of JSRV. The PCR products of three independent reactions were pooled and cloned in pGEM-T (Promega) as described in 2.3.10. Six selected clones (pGEM-T LTR-*gag*) were demonstrated to be of exogenous origin by the presence of a unique *ScaI* site in *gag* (data not shown). The region corresponding to the LTR of JSRV was then sequenced in both directions by the dideoxy chain termination method, employing a Li-Cor automated sequencer at the Sequencing Service facility of the Veterinary Pathology Department of the Royal (Dick) School of Veterinary Studies (University of Edinburgh, UK).

6.2.3 cDNA synthesis.

cDNA synthesis was carried out using total RNA derived from ultraspeed pellets of plasma (5 ml) or 50 µl of clarified lung fluid as template. For analysis of other samples, 2 µg of total RNA from tumour samples or 1 µg poly A⁺ RNA from other tissues and PBMC were used as template. RNA was diluted in 13.5 µl of diethyl pyrocarbonate-treated distilled water and denatured at 65°C for 10 min and cooled on ice. One microlitre (40 U) of rRNAsin (Promega), 2 µl of 25mM each deoxynucleoside triphosphate (dNTP), 2.5 µl of 100 mM dithiothreitol, 2.5 µl (A₂₆₀, 62.5 U/ml [Oswel] of random hexamers, 2.5 µl of 10X reaction buffer (500 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂) and 1 µl of MoMuLV reverse transcriptase (50U/µl -Stratagene) were added. Reverse transcriptase (RT) was omitted in control samples to check for DNA contamination of the RNA preparations. The reaction mixture was then incubated at 37°C for 1 h, and the RT inactivated by incubation at 94°C for 3 min.

6.2.4 Exogenous-specific JSRV U3 and U3 hemi-nested (hn) PCRs.

Primer P5 was paired with an antisense primer (P6, 5'-CACCGGATTTTACAC AATCACCGG-3') in the U3 region at nucleotide positions 7385-7361 where there are major sequence differences between JSRV and *enJSRVs*. In this region, the endogenous sequences carry a 30 bp insertion compared to the exogenous sequences (Fig. 6.1). The JSRV U3-PCR reaction was optimised using DNA from a LTR-*gag* pGEM-T plasmid as template. The buffer used was 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂ (pH 9.2) with 200 µM each dNTP, 6.25 pmol of each primer, 1.25 U of Taq polymerase (Boehringer) in a 50 µl reaction. Five hundred ng of genomic DNA or 3 µl of cDNA were used as template in the reaction. Each sample was subjected to 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 59°C for 1 min and 72°C for 1 min with a final extension 72°C for 3 min.

Total RNA extracted from 22 lung tumour and lung fluid samples from 16 different naturally acquired and 4 experimentally induced SPA cases were tested to verify the ability of JSRV-U3 PCR to detect different isolates of JSRV. In addition, genomic DNA extracted from lung tumour and matched kidney samples of four sheep with SPA was tested to verify that JSRV U3-PCR does not amplify *enJSRV* sequences.

To verify the specificity of the PCR products, twenty-five μ l of the PCR product was resolved by electrophoresis in 1.5% agarose gels and transferred to nylon membranes overnight by established procedures (Sambrook *et al*, 1989). Membranes were then fixed, pre-hybridised and probed overnight at 42 °C with 10 pmol each of two digoxigenin (DIG) 5'-labelled oligonucleotides (P-7, 5'-GGCACTGCTTCAC AGAAATATCAGGAAATCTGATT-3' [position 7316-7350]; P8, 5'-TTTTTAAA AGCTCTTAAGGCTCGGATGTTTGCTT-3' [position 7280-7313]) (Oswel) in Dig Easy Hyb (Boehringer) hybridisation buffer. Washings were performed in 0.1 X SSC at 52°C). The hybridised probe was then immunologically detected using the DIG Nucleic Acid detection Kit (Boehringer) as described in 2.3.9.

To improve the sensitivity of the JSRV-U3 PCR, a primer internal to the PCR product, primer P-9 (5'-TGATATTTCTGTGAAGCAGTGCC-3', position 7316-7338) was synthesised and paired with primer P-5 in a second round hemi-nested PCR (JSRV U3-hn PCR). One μ l of the product of the first round of amplification was added to 49 μ l of 1 X PCR buffer (1.25 U of *Taq* polymerase [Boehringer], 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl, 200 μ M each dNTP and 6.25 pmol of each primer). PCR cycles employed were 94°C for 1 min and 35 cycles of 94°C for 30 sec, 57°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 3 min. Amplified products were detected by electrophoresis of 25 μ l aliquots through a 2% agarose gel containing 0.5 μ g/ml of ethidium bromide.

The sensitivity of the JSRV U3-PCR and U3-hn PCR, against a background of endogenous-related sequences, were compared with each other and with the JSRV *gag*-PCR followed by *ScaI* digestion (described in chapter 4). 'Reconstruction' experiments were performed using serial dilutions (10^5 to 10^{-1} copies) of clone pGemT-LTR-*gag* (which contains the U3 through *gag* of JSRV provirus) with and

without the addition of 500 ng of normal sheep kidney DNA as a source of *enJSRV* sequences. In addition, 100 copies of pGEM-T LTR-*gag* plasmid, plus serial dilutions (10^2 - 10^7 copies) of *enJSRV*-LTR plasmids were tested.

6.2.5 Template control PCRs.

Each cDNA sample which was negative after the U3-hn PCR was tested for the presence of GAPDH transcripts to verify the integrity of the RNA template. Primers were designed on a consensus sequence between the rat and human GAPDH (C. McInnes, personal communication). The sense primer (5'-TCACCACCATGGAG AAGGCT-3') was based on exon 4 of the human gene and the reverse primer was based on exon 7 (5'-TTCATTGTCATACCAGGAAA-3') (Tokunaga *et al*, 1987). The buffer employed was 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂ (pH 8.9) with 200 μ M each dNTP, 6.25 pmol of each primer, 1.25 U of Taq polymerase (Boehringer). Cycles employed were 94 °C for 1 min and 35 X (94°C for 45 sec, 52°C for 1 min and 72 °C for 1 min and 30 sec with a final extension of 72°C for 5 min).

Parallel samples in which the addition of RT was omitted in the cDNA synthesis step were also tested to rule out DNA contamination. Amplified products were detected by electrophoresis of 25 μ l aliquots through 1% agarose gels in 1X TBE buffer in the presence of 0.5 μ g/ml of ethidium bromide.

A DIG-labelled internal probe (5'-DIG-CTCATGACCACAGTCCATGCCATCAC GCC-3') was used to confirm the specificity of the amplified product. Southern blotting and detection of hybridised probe was essentially as described above for U3-PCR.

DNA samples which were negative after hn-U3 PCR were checked by amplification of a portion of *enJSRVs gag* as described in Chapter 4.

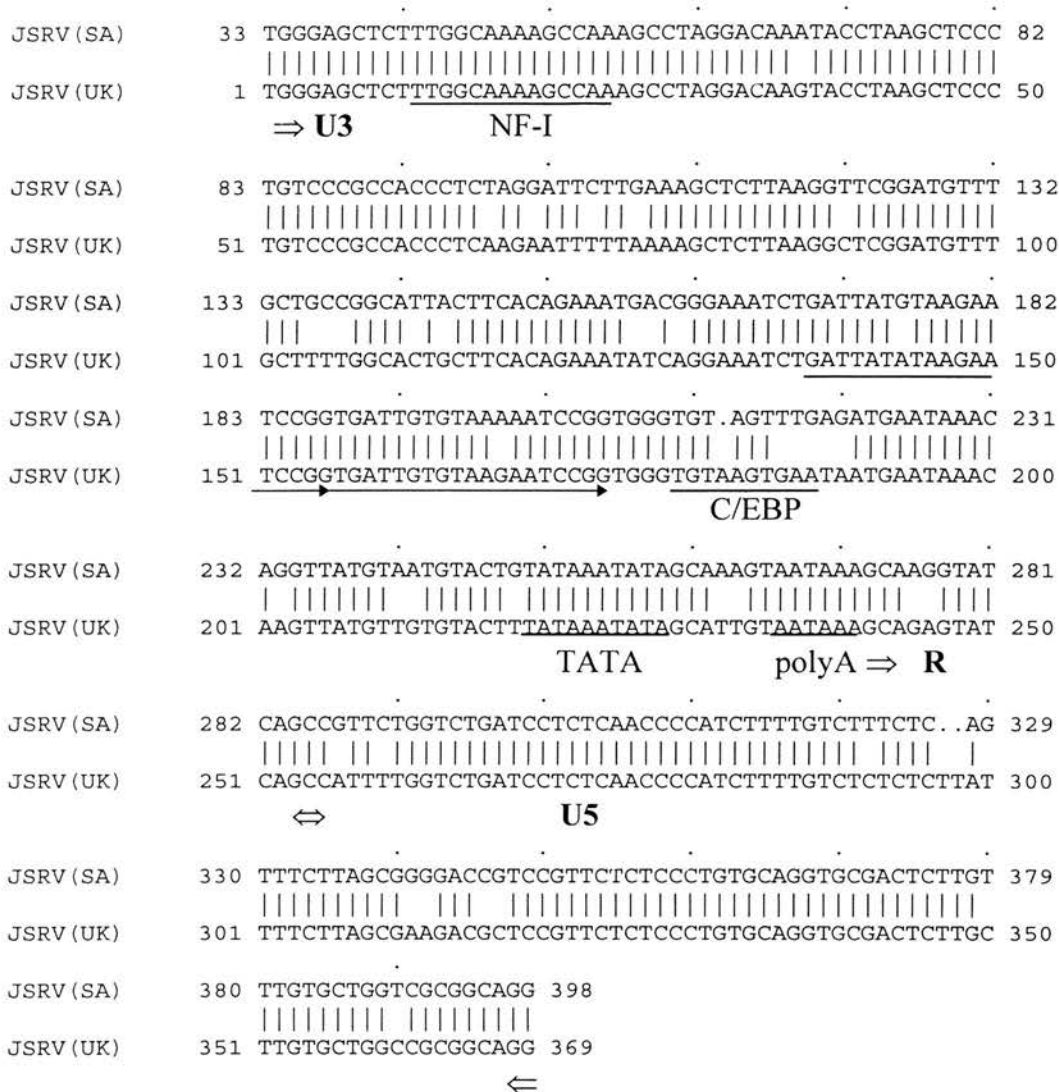


Fig. 6.1. Sequence alignment of the LTR of the South African isolate [JSRV(SA)] (York *et al*, 1992) and of the UK isolate of JSRV [JSRV(UK)]. Enhancer- like sequences, TATA box and the poly adenylation sites are indicated. A tandem repeat is underlined by arrows (→). Open arrows (⇒) indicate limits of U3, R and U5.

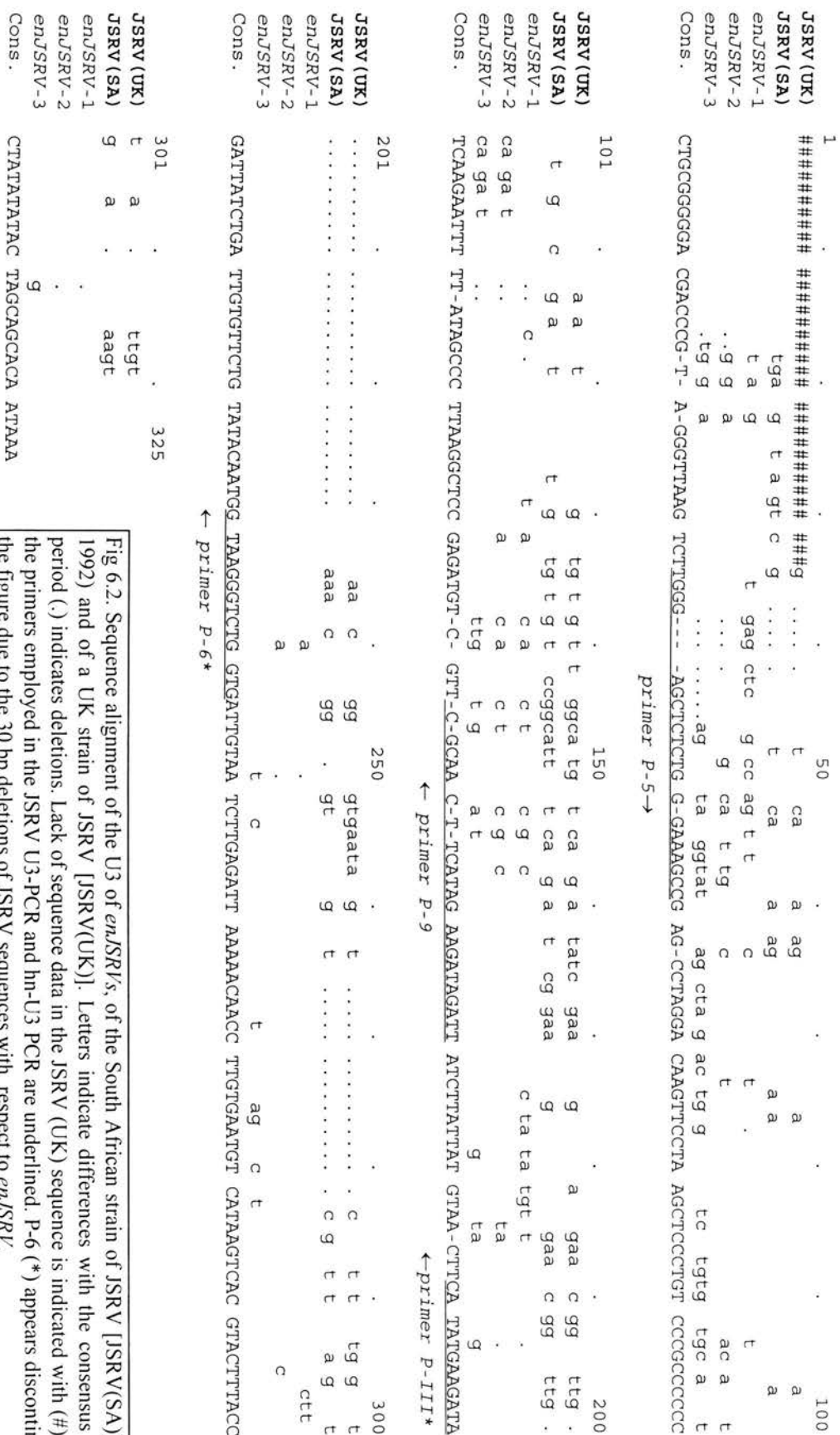


Fig 6.2. Sequence alignment of the U3 of *enJSRV*s, of the South African strain of JSRV [JSRV(SA)] (York *et al.*, 1992) and of a UK strain of JSRV [JSRV(UK)]. Letters indicate differences with the consensus sequence. A period (.) indicates deletions. Lack of sequence data in the JSRV (UK) sequence is indicated with (#). Positions of the primers employed in the JSRV U3-PCR and hn-U3 PCR are underlined. P-6 (*) appears discontinuous only in the figure due to the 30 bp deletions of JSRV sequences with respect to *enJSRV*.

6.3 Results.

6.3.1 Sequencing of JSRV LTR and designing of JSRV exogenous-specific primers.

To better design an exogenous specific-PCR the LTR of a UK strain of JSRV was sequenced and compared with the sequence of a South African strain of JSRV and to the LTR of *enJSRVs* (York *et al*, 1992; Palmarini *et al*, 1996). The LTR of the UK strain of JSRV [JSRV(UK)] was 89% homologous to the LTR of the South African strain [JSRV(SA)] (Fig. 6.1) (York *et al*, 1992) and 76 to 79% homologous to *enJSRV* loci (Fig. 6.2). Major sequence divergence between exogenous and endogenous viruses was confirmed in the U3 region. In particular, a 30 bp deletion in the U3 of JSRV(SA) compared to the endogenous loci was also confirmed in JSRV(UK). Primer P-6 was designed across the deletion such that at the optimal annealing temperature this primer should anneal efficiently only with the exogenous sequences. The alignment of the U3 of JSRV and *enJSRVs* sequences and the relative positions of the primers employed in this study are indicated in Figure 6.2.

The JSRV LTR sequence of the UK strain has been deposited in GenBank under accession number Z71304.

6.3.2 Specificity and Sensitivity of JSRV U3 and hemi-nested-U3 PCRs.

Twenty-two cDNA samples of lung fluid and lung tumour were examined by PCR using primers P-5 and P-6. A product of the expected size of 176 bp was obtained with all samples. To demonstrate the PCR product was derived only from JSRV and not from *enJSRV*, matched genomic DNA samples of lung tumour and kidney from four SPA-affected animals were examined. An amplified product was obtained from tumour DNA and not from the corresponding kidney sample, proving that the JSRV-U3 PCR is exogenous-specific (Fig. 6.3 [top]). Hybridisation with the DIG-labelled

internal oligonucleotides P-7 and P-8 confirmed that the products originated from JSRV-LTR (Fig. 6.3 [bottom]).

The sensitivity of the U3-PCR was determined by 'reconstruction' experiments, testing mixtures of exogenous JSRV and *enJSRV* templates described in Materials and Methods. JSRV U3 PCR successfully amplified 10^2 - 10^3 JSRV template copies in a background of 500 ng of normal sheep genomic DNA and 10^2 JSRV template copies in a background of 10^7 copies of *enJSRV*-LTR.

The sensitivity of the U3-PCR was increased by using primer P-6 and primer P-5 in a second round PCR. A product of 133 bp was detected from the re-amplification of all the lung fluid and lung tumour PCR products but not from the kidney samples. The U3-hn PCR detected an estimated single molecule of template in a background of 500 ng of normal sheep genomic DNA (Fig. 6.4). In contrast, as described in chapter 4, PCR-*gag* followed by *ScaI* digestion was at least 10^5 times less sensitive than the U3-hn PCR because 10^5 copies of exogenous clone were not detected against a background of 500 ng of normal sheep kidney DNA. Furthermore exogenous sequences were detected by the U3-hn PCR only when the ratio of exogenous/endogenous sequences was 1:10 but not $\geq 1:100$.

The U3-hn PCR amplified only exogenous sequences and was specific for all strains of JSRV tested.

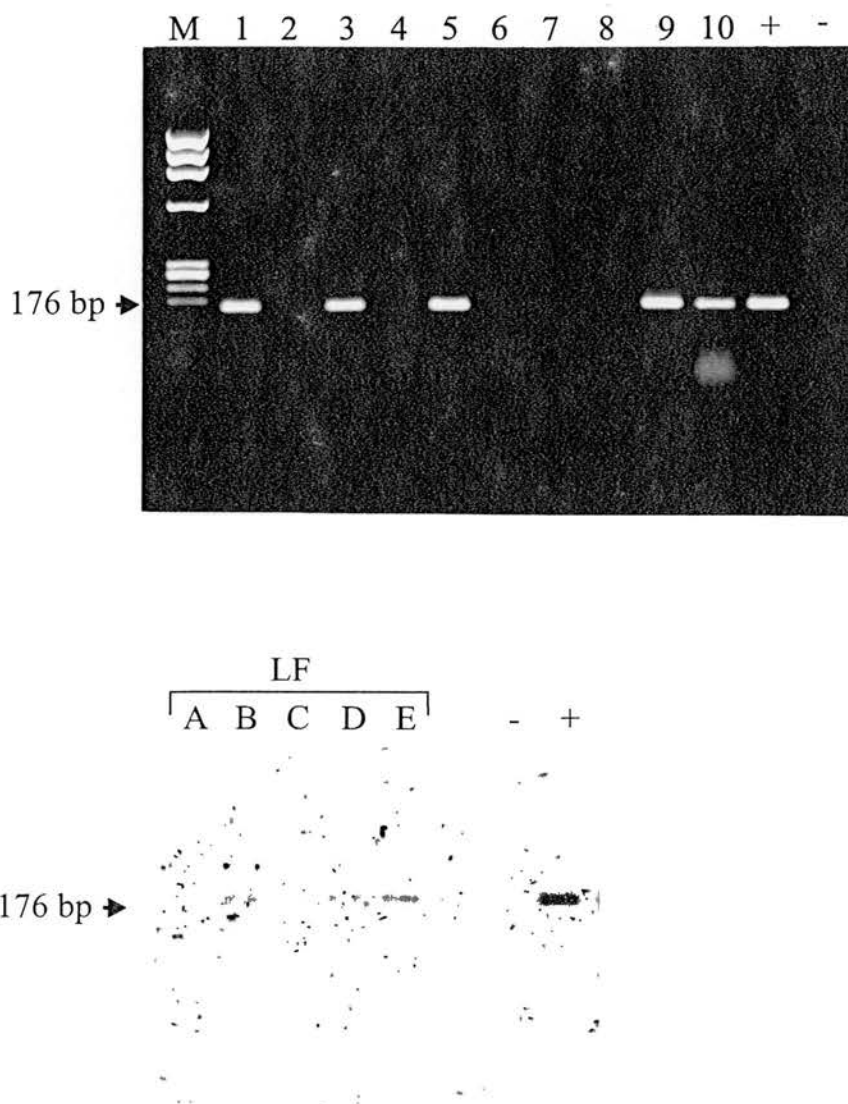


Fig. 6.3: **Specific amplification of JSRV by U3-PCR.** (Top panel) Lanes 1, 3 and 5 are the product of amplification of 500 ng of lung tumour genomic DNA from three cases of SPA; lanes 2, 4 and 6 are the matched kidney samples. Lanes 7 and 8 are cDNA of lung of healthy control sheep; lane 9 is lung tumour cDNA, and lane 10 is lung fluid cDNA. + and - indicate pGEM-T LTR-*gag* and water respectively used as positive and negative controls. Lane M contains molecular weight marker IX (Boehringer). (Bottom panel) Southern hybridisation of lung fluid samples from five SPA cases (lanes A-E) and probed with DIG-labelled probes P7 and P8. + and - indicate pGEM-T LTR-*gag* and water respectively used as positive and negative controls.

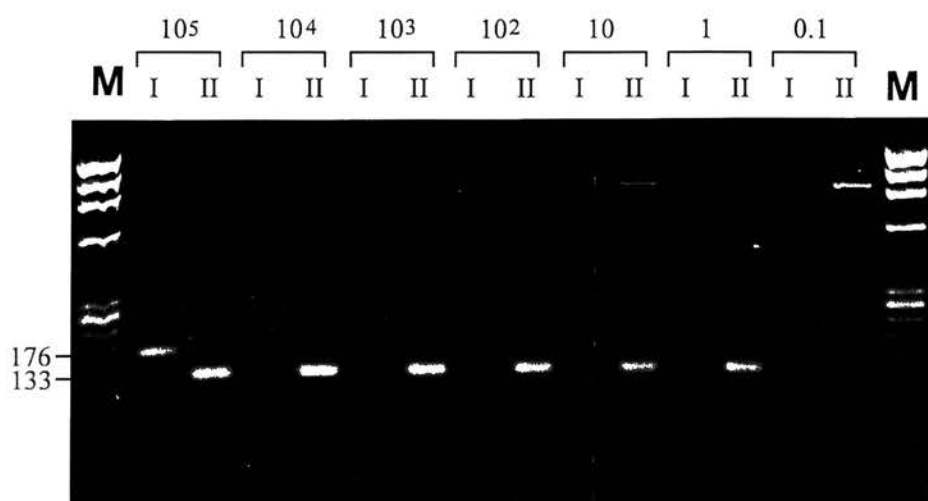


Fig. 6.4: **Sensitivity of JSRV U3-hn PCR.** Serial dilutions of pGem-T LTR-*gag* containing the JSRV LTR template were amplified with a background of 500 ng of sheep genomic DNA collected from an unaffected control sheep. The number of copies is indicated at the top of the gel. Lanes: I, first step of JSRV U3-hn PCR; II, second step of U3-hn PCR; M, molecular weight marker IX (Boehringer).

6.3.3 Detection of proviral DNA and JSRV transcripts in non-tumour tissues of SPA-affected sheep.

Following the successful development and validation of the U3-hn PCR, the anatomical distribution of JSRV in SPA-affected sheep was analysed. Table 6.1 summarises the results obtained.

JSRV RNA and proviral DNA was found consistently in the mediastinal lymph nodes of SPA-affected sheep (10/10). In addition, JSRV-RNA was found in 7/9 spleens, 4/8 bone marrows, and in 2/4 thymuses (Fig.6.5). Proviral DNA was found more rarely in these tissues. PCR products were detected in 2/8 spleens, and 3/7 bone marrows. Among the non-regional lymph nodes analysed all were negative for proviral DNA, but two mesenteric lymph nodes of the five analysed were positive at the RNA level. Pre-scapular lymph nodes (n=4) were always negative. PBMC were found positive for JSRV RNA in the 3/7 samples but proviral DNA was not detected in any. Plasma samples were always negative.

Apart from lymphoid tissues, JSRV was detected at both the DNA and RNA level in only one kidney of 9 tested and in none of four skin samples. No differences were observed in the anatomical JSRV distribution between the naturally-acquired and the experimentally-induced SPA cases.

JSRV was not detected in 27 samples examined from 15 unaffected control animals. These samples included polyA⁺ RNA prepared from lung (n=5), mediastinal lymph nodes (n=7), thymus (n=1) and PBMC (n=4). In addition, samples from lung (n=3), mediastinal lymph nodes (n=1), spleen (n=2), thymus (n=1), bone marrow (n=1) and kidney (n=2) were tested for proviral DNA.

All the samples that were negative in the U3-hn PCR were positive for the amplification of GAPDH cDNA or *enJSRV gag* DNA, demonstrating that the templates were not degraded, and PCR inhibitors were not present after the nucleic acid extraction.

Table 6.1. Results of the JSRV U3-hn PCR on tissues of sheep affected by SPA.

| TISSUES | Natural SPA* | | Experimental SPA† | | TOTAL | |
|-------------------------|---------------------|--------------------|-------------------|-------------------|-------------------|-------------------|
| | RNA‡ (+/tested)¶ | DNA§ (+/tested) | RNA (+/tested) | DNA (+/tested) | RNA (+/tested) | DNA (+/tested) |
| Lung tumour/lung fluid | 18/18 | 6/6 | 4/4 | 4/4 | 22/22 | 10/10 |
| Mediastinal lymph nodes | 6/6 | 6/6 | 4/4 | 3/3 | 10/10 | 9/9 |
| Other lymph nodes | 2/9 | 0/9 | na | na | 2/9 | 0/9 |
| Spleen | 4/5 | 0/5 | 3/4 | 2/3 | 7/9 | 2/8 |
| Bone marrow | 2/5 | 2/4 | 2/3 | 1/3 | 4/8 | 3/7 |
| Thymus | na | na | 2/4 | 0/4 | 2/4 | 0/4 |
| Kidney | 1/6 | 1/6 | 0/3 | 0/3 | 1/9 | 1/9 |
| Skin | 0/4 | 0/4 | na | na | 0/4 | 0/4 |
| Plasma | 0/2 | / | 0/6 | / | 0/8 | / |
| PBMC | 3/7 | 0/6 | na | na | 3/7 | 0/6 |

*Sheep with naturally acquired SPA
†Sheep with experimentally induced SPA
‡polyA⁺ selected RNA
§ genomic DNA
¶ (number of positive samples/number tested)
na = not available

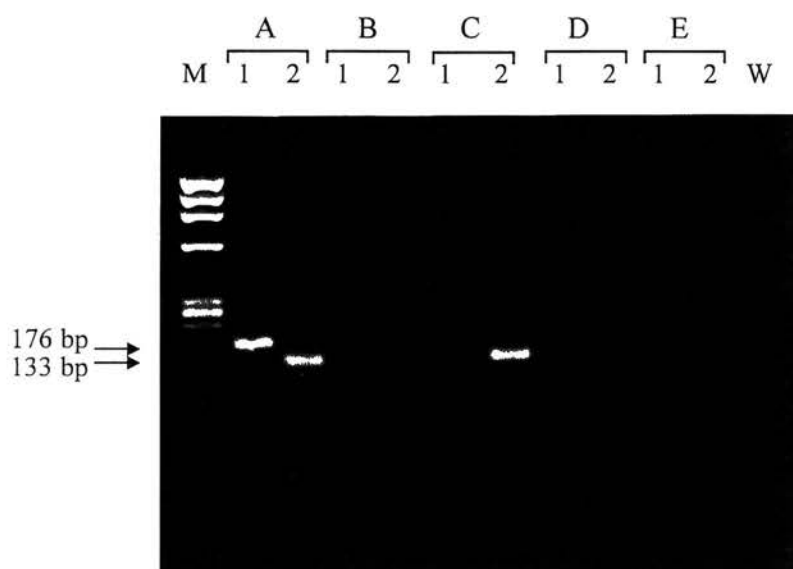


Fig. 6.5: Example of the results of JSRV U3-hn PCR in an SPA-affected animal. 1, indicates the first step of the U3-hn PCR while 2 represents the second round of the reaction. Lane A, lung tumour cDNA; lane B, kidney cDNA; lane C, spleen cDNA (note the faint product obtained in the first step); lane D skin cDNA; lane E, lung cDNA from an unaffected control animal. Lane M contains molecular weight marker IX. W is the negative control (water) of the hn-U3 PCR.

6.4 Discussion.

Studies described in the previous chapters, using immunological and molecular techniques, demonstrated that JSRV is an exogenous virus, which does not arise from reactivation of an endogenous locus. These studies identified the lungs, particularly the epithelial tumour cells, as a major site of JSRV replication, although viral RNA was demonstrated in the draining mediastinal lymph nodes of a proportion of SPA-affected sheep. These observations have been extended in the present study as a result of developing a highly sensitive assay for exogenous JSRV and show that JSRV RNA and proviral DNA is present not only in the draining lymph nodes but also in several anatomically dispersed lymphoid tissues and PBMCs. This distribution of JSRV in infected animals is, therefore, similar to that reported for other type B and type D retroviruses, in which both lymphoid and non-lymphoid tissues are infected (Bentvelzen & Brinkhof, 1980; Kozma *et al*, 1980; Bryant *et al*, 1986; Lackner *et al*, 1988).

Although quantitative assays were not employed in the present studies, the estimated sensitivity of the different PCR assays confirmed that JSRV was expressed at much higher levels in SPA tumours than in the lymphoid tissues, particularly those at the non-local sites. Thus, there are clear parallels with the high levels of MMTV transcription associated with the development of breast carcinoma in mice (Varmus *et al*, 1973; Bramblett *et al*, 1995). The transcriptional activity of MMTV appears to be determined by the interaction of a number of factors involving regulatory elements within the LTR, including at least one region implicated in elevated transcription in the mammary epithelium (Lefebvre *et al*, 1991; Mink *et al*, 1992; Mok *et al*, 1992), and the absence of tissue-specific factors such as NBP in mammary tissue (Bramblett *et al*, 1995). Similar putative regulatory sequences, such as NF-1 and C/EBP binding sites, have been identified in the JSRV LTR (York *et al*, 1992) as well as many point mutations and large deletions compared to endogenous loci as described in Chapter 5. Other retroviruses with LTR deletions have been reported to

have a transcriptional advantage (Hsu *et al*, 1988) and the biological significance of these structures in JSRV awaits the development of permissive culture systems.

The interaction between type B and type D retroviruses and lymphoid cells has been demonstrated to be an important feature in the pathogenesis of these viruses. B lymphocytes have a central role in the pathogenesis of MMTV and infection of the mammary gland requires a functional immune system. MMTV initially appears to infect B lymphocytes and is then delivered to the mammary gland through T-B cell interactions (Tsubura *et al*, 1988; Golovkina *et al*, 1992; Held *et al*, 1994). The simian type D retrovirus, SRV 1, which induces simian acquired immunodeficiency syndrome in macaques (Gardner *et al*, 1994), has a broad *in vivo* lymphoid tropism involving lymphocytes and mononuclear cells. Infection leads to both T and B lymphocyte depletion although monocyte and macrophage function appears unaffected (LeGrand *et al*, 1985; Maul *et al*, 1988).

The significance of infection of lymphoid tissues by JSRV in the overall pathogenesis of SPA is unknown at present. As only sheep with clinical SPA were examined it is not known whether lymphoid infection precedes, or is a sequel to, JSRV replication in the pulmonary epithelium. Further studies will be required to define which cell types are involved and at which stages during the protracted time between infection and the appearance of clinical illness. Similarly, it is not clear whether sheep infected with JSRV develop immunodeficiency, although this possibility is supported by anecdotal reports of the increased susceptibility to secondary bacterial pneumonias (Sharp and Martin, 1983; Verwoerd, 1990) and a lymphopaenia in SPA-affected sheep (Rosadio and Sharp, 1992). The apparent absence of circulating antibodies to JSRV in sheep with SPA has been reported (Sharp and Herring, 1983; Verwoerd, 1990) although a recent study, using a recombinant Mason-Pfizer monkey virus major capsid protein, has produced conflicting results (Kwang *et al*, 1995).

One of the major obstacles to progress in research on JSRV has been the lack of an *in vitro* culture system. The present study has clearly demonstrated permissive infection of lymphoid cells by JSRV, cell types which have been used successfully for the

propagation of many retroviruses and which, therefore, could provide a permissive substrate for JSRV replication.

Chapter 7.

General Discussion and Conclusions.

7.1 Summary of the results obtained and general discussion.

In this final chapter, the results obtained during the course of this project will be summarised and discussed taking into account also results obtained by other investigators in the last three years. Important areas of research on SPA where almost no data are available at the moment, such as the immune response towards JSRV or the mechanisms of oncogenesis, will also be discussed. Possible approaches towards the understanding of these events will be proposed.

- **Sites of JSRV replication.**

Studies on the aetio-pathogenesis of SPA were hampered for a number of years by the lack of immunological reagents and techniques to detect JSRV, the major candidate to be the aetiological agent of SPA. After the sequencing of one strain of JSRV (York, *et al* 1992), new possibilities for research were created.

The sites of viral replication were among the first aspects to be investigated in this project. In Chapter 3, it has been described how immunological reagents and techniques for the detection of JSRV were developed and then applied to investigate the sites of JSRV translation and assembly. Using a JSRV-specific blocking-ELISA, viral particles were detected only in lung fluid and tumour extracts of SPA-affected sheep. The infected cells were identified by immunohistochemistry as the recognisable transformed epithelial cells (Palmarini *et al*, 1995). Employing these techniques, JSRV was not detected outside the tumour and its secretions in SPA-affected sheep and was never detected in unaffected control animals.

The detection of JSRV constantly and exclusively in SPA-affected sheep suggested a causal association between this virus and the onset of the neoplastic sequelae. However, the presence in the sheep genome of JSRV-related endogenous retroviruses complicated a straightforward interpretation of the results obtained. No information was available to explain the relationship between the exogenous JSRV, transmitted

with the lung fluids by SPA-affected sheep, and its endogenous counterparts (*enJSRVs*).

- **JSRV is distinct from its endogenous counterparts.**

In Chapter 4, it was shown that *enJSRVs* are not only present in the sheep genome but are transcriptionally active in a wide variety of tissues in both normal and SPA-affected sheep (Palmarini *et al*, 1996a). It was, therefore, necessary to establish that JSRV was not an endogenous virus. At that stage, different scenarios could have been envisaged:

- i) SPA could be induced by JSRV, alone or in association with unknown co-factors, through horizontal transmission between sheep;
- ii) JSRV could be the “helper” virus of an uncharacterised acutely transforming retrovirus (the primary cause of SPA);
- iii) SPA could be induced by the reactivation of one of the *enJSRVs*, in a similar way to the induction of mammary tumours in the European GR strain of mice. Here, a single endogenous virus which behaves as a dominant locus, *Mtv-2*, induces both the early appearance of mammary tumours and the presence of MMTV virions in milk (Muhlbock and Bentvelzen, 1969; Van Nie *et al*, 1977; Morris *et al*, 1986);
- iv) SPA could be caused by an unidentified infectious agent and the presence of JSRV in tumour cells could be only “accidental” and not necessarily linked to the induction of neoplasia as for example the presence of endogenous RD-114 in feline lymphoid tissues and neoplasms (Niman *et al*, 1977a, b and 1980).

In order to investigate these different hypotheses it was first necessary to characterise JSRV and its endogenous counterparts.

As described in Chapter 4, by sequencing part of the *gag* gene of *enJSRVs*, a *ScaI* restriction site was established as a molecular marker of the exogenous JSRV. Restriction enzyme digestion of *gag*-PCR products obtained from several lung fluid

samples from Scotland and Spain confirmed that *ScaI* was indeed present in different isolates of JSRV in addition to the South African and Peruvian isolates (York *et al*, 1992; Hecht *et al*, 1994). This molecular marker was subsequently found to be conserved in different isolates in South Africa (D. York, personal communication), Spain (M. Garcia, personal communication), USA and Kenya (Bai *et al*, 1996). In addition, major differences between JSRV and *enJSRVs* were found in the LTRs and in particular in the U3 region (Palmarini *et al*, 1996a; Bai *et al*, 1996). In all retroviruses this region of the LTR contains the transcriptional promoter and enhancer elements. Sequence variations in the enhancer region affect retroviral expression, tissue-specificity and oncogenicity (Des Groseillers *et al*, 1983; Weber and Schaffner, 1985; Cullen *et al*, 1985; Stocking *et al*, 1986; Ishimoto *et al*, 1987; Golemis *et al*, 1989; Ryden *et al*, 1989; Greuel *et al*, 1990; Speck *et al*, 1990; Hallberg *et al*, 1991; Lefebvre *et al*, 1991; Mink *et al*, 1992; Mok *et al*, 1992). Therefore, it is not surprising to find variations in the U3 between JSRV and *enJSRVs*. Differences in the U3 between pathogenic exogenous retroviruses and non-pathogenic endogenous counterparts have been shown, for example, in the avian leukosis virus group between the non-pathogenic endogenous RAV-0 and the leukemogenic RAV-1 (Robinson *et al* 1982 and 1985; Brown *et al*, 1988). Chimaeric constructs indicated that the RAV-1 LTR was necessary for the viral leukaemogenic potential, although other viral genes also were important. Similar examples have been described in some MuLVs (DesGroseillers *et al* 1983; Lenz and Haseltine, 1983; DesGroseillers and Jolicoeur, 1984; Lenz *et al* 1984).

The differences between JSRV and *enJSRVs* LTR were made use of to develop a PCR assay for the detection of JSRV at DNA level (Chapter 5). The detection of exogenous proviral DNA in tumour but not in matched kidney DNA from SPA-affected animals (Palmarini *et al*, 1996a) proved that JSRV is an exogenous horizontally transmitted virus and does not derive from the reactivation of an endogenous virus.

The finding that some *enJSRVs* are transcriptionally active does suggest the possibility that the exogenous virus could have recombined with some *enJSRVs*. The

contemporary presence of exogenous and endogenous transcripts in the neoplastic (or pre-neoplastic) cells could lead to copackaging of the two different molecules. Recombination would then occur by reverse transcriptase switching during DNA synthesis in the following round of infection. Retrovirus recombination is a well established mechanism and can occur either after co-infection of two distinct exogenous retroviruses or between an exogenous and an endogenous virus (Goodrich and Duesberg, 1990; Hu and Temin, 1990; Coffin 1990b).

Although recombination between exogenous and endogenous viruses cannot be ruled out, it was important that JSRV and *enJSRVs* were found to be distinct entities and that JSRV was found specifically associated with SPA tumours. The establishment of JSRV molecular markers allowed, for the first time, studies on the virus-host interaction at a molecular level.

- **JSRV establishes a disseminated infection of the lymphoid tissues.**

The designing of JSRV exogenous-specific primers and the development of a sensitive exogenous-specific PCR allowed further investigation of the sites of JSRV replication and in this way identification of novel sites of JSRV replication.

JSRV was demonstrated to establish a disseminated infection of the lymphoid tissues of SPA-affected sheep (Chapter 6; Palmarini *et al*, 1996b). The mediastinal lymph nodes, which drain the lungs, were shown consistently to be infected by JSRV. JSRV transcripts were detected also in a proportion of spleen, thymus, bone marrow and peripheral blood mononuclear cells. Proviral JSRV DNA also was detected in these tissues, although in a much lower proportion of cases (Palmarini *et al*, 1996b). Preliminary results indicate that JSRV infects both adherent cells (mainly macrophages) and lymphocytes (B and T-cells [CD4⁺ and CD8⁺]). These data were obtained employing the U3-hn PCR (described in chapter 6) in MACS sorted mediastinal lymph nodes cells from two terminal cases of SPA. Quantitative analysis of the samples by limiting dilution analysis estimated a higher proviral load in the “adherent” cells (one provirus for 3,500 to 5,400 cells) compared to the lymphocytes

(one provirus per 9,300 to 42,136 cells) (Palmarini M., Holland M., Dalziel R.G. and Sharp J.M, unpublished results). However, more samples need to be analysed to confirm these data.

- **Is the dissemination of JSRV in the lymphoid tissues significant in the pathogenesis of SPA?**

It is difficult to answer this question when only scant data about the immune response towards JSRV are available.

SPA-affected sheep do not show an appreciable humoral immune response towards JSRV. No circulating antibodies have been detected by western blotting when a ultracentrifuged pellet of lung fluid was probed with SPA-affected sheep sera (Sharp and Herring, 1983; Verwoerd, 1990). Apparently contrasting results have been obtained by indirect ELISAs based on recombinant CA protein of MPMV or of ENTV coupled to GST. The use of these tests showed differences in the percentage of sero-positivity between flocks with a history of SPA or chronic respiratory diseases and flocks where SPA was never described (Kwang *et al*, 1995; Rosati *et al*, personal communication). However, the interpretation of these results is doubtful. No major differences were obtained in a blind trial when sera of SPA-affected sheep were compared with sera from healthy control sheep (included sheep from the Falkland Islands where SPA has never been described (A. Coe, personal communication) (S. Rosati, M. Palmarini and J.M. Sharp, unpublished data). The presence of antibodies in SPA-affected sheep has therefore to be further investigated. Assuming that the results obtained by western blotting truly reflect the lack of a humoral immune response towards JSRV, it could be hypothesised that SPA-sheep are immunotolerant towards JSRV. However, the lack of circulating antibodies in a retroviral infection does not necessarily reflect the lack of an immune response. For example, an HIV-specific cytotoxic T cell response in HIV-exposed and seronegative Gambian sex workers has been recently described (Rowland-Jones *et al*, 1995).

It is useful however to speculate on possible tolerance in sheep towards JSRV as this would have important biological implications.

Immunotolerance of sheep to JSRV could be explained as a result of expression of *enJSRVs* during ontogeny. In this case endogenous viral proteins would be recognised as “self” and induce the clonal deletion of lymphocyte precursors specific for the virus. Because the degree of self tolerance reflects the concentration of antigen reaching the thymus (Sprent and Webb, 1995) sheep would be more or less tolerant towards JSRV depending on the level and sites of *enJSRVs* expression.

Results in contrast with this hypothesis have been obtained by Sharp (unpublished data) who induced an antibody response in sheep immunised with recombinant CA-JSRV suspended in incomplete Freund’s adjuvant. However, it is known that tolerance to natural self-antigens, such as insulin and thyroglobulin, can be broken by an “unnatural” stimulus such as the injection of a high concentration of antigen suspended in adjuvant (Romball and Weigle, 1984; Whiteley *et al*, 1990).

The induction of tolerance due to the expression of endogenous retroviruses has been shown in other models. M-MuLV is integrated into the germline of a particular strain of mice (C57BL/6 Mov-13) and is expressed during the ontogeny in lymphoid and non-lymphoid tissues. When such mice are infected with M-MuSV, no antibody or CTL response is seen and a high incidence of T-cell lymphoma occurs (Ronchese *et al*, 1984). Another example is given by studies conducted by McMahon *et al* (1986) in the RSV system. In the FP line of chickens there are high levels of expression of an endogenous retroviral envelope glycoprotein (subgroup E) (Weiss *et al*, 1971). When these chickens are infected with either a subgroup B strain of RSV (which cross-react with subgroup E) or with a subgroup G (which does not cross-react) they develop a sarcoma at the wing web site. However, a high percentage of only the subgroup B-infected chickens exhibit distal sarcomas and they do not develop antibodies towards the envelope subgroup B protein in contrast to the substantial titres generated in the subgroup G. This difference is explicable on the basis of the tolerogenicity of endogenous viral glycoprotein.

In the SPA model the lack of an immune response could also be due to a viral-induced immunosuppression. A possible immunosuppressive role has been hypothesised for JSRV due to the fact that SPA-affected sheep are increasingly susceptible to secondary bacterial pneumonias (Sharp and Martin, 1983; Verwoerd, 1990) and to a report of lymphopenia in these animals (Rosadio and Sharp, 1992). These last observations have been made in a small number of sheep at the terminal stage of the disease. The extensive lesions in the lungs, the frequent secondary bacterial infections and the consequent respiratory impairment could be, by themselves, the cause of immunosuppression.

Epidemiological data suggest that some form of “resistance” in sheep develops after SPA is introduced for the first time in a particular territory. In endemic areas SPA has an incidence between 1 and 5% (see 1.2) but when SPA was first introduced in Iceland there were losses in some flocks between 50 and 80% in the first two years after which it gradually decreased (Dungal *et al*, 1938). An analogous situation happened in Kenya (Shirlaw, 1959; Wandera, 1967) and in Peru (Rosadio, personal communication) where the disease spread in an epidemic fashion before subsequently dropping. The initial epidemic spread and the subsequent decline of the incidence of an infectious disease could be attributed either to a protective immunity that arises in the susceptible population or natural selection of a less virulent strain of the infectious agent.

Appropriate recombinant proteins are much needed to evaluate the humoral and moreover the cellular immune response towards JSRV.

- **Which cells are first infected by JSRV?**

Infection by JSRV is thought to spread horizontally among sheep through the respiratory route and therefore through the inhalation of aerosol or droplets of lung fluid containing JSRV by a susceptible sheep in close contact with an SPA-affected sheep. No data support a transmission from mother to foetus. JSRV was never detected in lung, thymus and spleen collected from foetuses (n=3) derived from

sheep with SPA and analysed by JSRV U3-hn PCR (Palmarini, M., and Sharp, J.M., unpublished data).

The horizontal transmission through the respiratory route is confirmed by the sites of the tumours in naturally arising SPA-cases. Lesions are localised predominantly in the apical lobes of the lungs. Further evidence supporting the respiratory route of infection is given by the experimental model of SPA: lambs are successfully infected only by the intratracheal inoculation of lung fluid.

It is conceivable that, initially, JSRV infects the epithelial cells of the lungs where it is able to replicate actively and eventually transform these cells. After a few rounds of replication, antigen presenting cells (APCs) could phagocytose JSRV and carry the virus to the mediastinal lymph nodes draining the lungs where JSRV could be presented more or less efficiently to lymphocytes and then through the recirculation of these cells be disseminated to other lymphoid tissues.

If the infection of the lymphoid cells with JSRV happens after the infection and transformation of the epithelial cells it could be hypothesised that it is not a necessary step for the virus to induce disease but more probably a consequence of the immune system of the host to respond to the viral infection.

As has been shown previously (chapter 6; Palmarini *et al*, 1996b) both JSRV RNA and proviral DNA have been detected in lymphoid cells and this suggest that the virus is able to perform at least the first steps of its replication cycle in these cells.

It is possible that JSRV infection of the lymphoid cells precedes JSRV replication and transformation of the epithelial cells of the lungs. In this case the infection of the lymphoid cells could be seen as a “necessity” for JSRV to induce transformation of type II pneumocytes.

This hypothesis is based on some characteristics which are shared between the JSRV and MMTV systems. Like JSRV, MMTV is associated with an epithelial neoplasm which represents the main site of viral replication. In addition, MMTV causes a disseminated infection of the lymphoid tissues where low levels of viral replication are detectable (Matsuzawa *et al*, 1995).

MMTV exploits the immune system to reach the mammary gland and induce tumour. The infectious MMTV is transmitted through the milk from mother to baby and taken up by the neonatal gut epithelium where viral proteins are detectable. It then infects B cells of the Peyer's patches which (Karpetian *et al*, 1994) stimulate a large proportion of T cells of the animal through the MMTV Sag (Acha-Orbea and MacDonald, 1993; Held *et al*, 1994; Huber, 1995). T cells (carrying the Sag-specific V_{β} region) then provide a positive feed-back (by cytokine production) for the few infected B cells to proliferate and in this way expand MMTV infection (Waanders *et al*, 1993) which finally reaches the mammary gland. Mice lacking B-cells cannot be infected (Beutner *et al*, 1994). In mice lacking Sag-reactive T-cells, viral amplification does not occur which results in a loss of the virus before it can reach the mammary gland of lactating females and be transmitted to the offspring (Tsubura *et al*, 1988; Golowkina *et al*, 1992; Held *et al*, 1993a). MMTV therefore requires an intact immune system to be able to reach the mammary gland which is the only place where virus production has been clearly detected (about 10^{12} particles per ml of milk; Acha-Orbea, 1996).

However, the pathogenesis of MMTV-induced mammary carcinoma and SPA differs in an important aspect: the sites of entry of the two viruses with respect to the accessibility of their target cells for transformation. While MMTV enters through the digestive tract and then has to "travel" to reach the mammary gland, JSRV enters through the respiratory route and therefore type II pneumocytes and the Clara cells are readily accessible, although a previous transit of JSRV in the lymphoid tissues of the upper respiratory tract (e.g. tonsils) cannot be ruled out.

In addition, MMTV Sag expression plays a central role in the interaction with the immune system of the host and no data are available in this respect for JSRV.

Analysis of the sites of JSRV replication in the early stages of the infection might elucidate the temporal relationship between infection of the epithelial cells, infection of the lymphoid cells and transformation.

- **What are the mechanisms of oncogenesis?**

The mechanisms by which JSRV transforms its target cells are unknown and can only be speculated on at the present time.

JSRV has the genomic organisation of a replication competent retrovirus with no oncogenes. Thus, a likely possibility is that JSRV transforms type II pneumocytes by insertional mutagenesis. To test this hypothesis it will be necessary to analyse the sites of viral integration in several SPA tumours collected from different affected animals and verify if common integration sites occur.

Tumours which arise by insertional mutagenesis usually have a long incubation period (see 1.7.2; Teich, 1984; Fan, 1994) because retroviral integration is considered essentially a random event (Luciw and Leung, 1992) and multiple replicative cycles will have to occur before the virus integrates near an oncogene. In addition, this is generally only one of a series of events which have to occur before cell transformation is triggered.

The incubation period in naturally-acquired SPA is not known with certainty. However, on the basis of old epidemiological studies (Sigurdsson, 1954) and on the observation that in nature SPA is a disease affecting mainly adult sheep (Sharp and Martin, 1983; Verwoerd, 1990) the incubation period seems to be ranging from months to years. Therefore, naturally acquired SPA seems therefore to fit the insertional mutagenesis model.

In the experimentally-induced SPA however, the clinical manifestations of SPA are achieved in approximately four weeks by intratracheal inoculation of lung fluid in new-born lambs (Verwoerd *et al*, 1980; Sharp *et al*, 1983; DeMartini *et al*, 1987). Such short incubation periods are generally seen in tumours induced by acutely transforming retroviruses (Bishop and Varmus, 1984; Rasheed, 1995) and not by retroviruses which act by insertional mutagenesis. However, it is only possible to reproduce efficiently the disease with this short incubation period only when new-born lambs are used for the experimental infection. Experimental infection of lambs

of 10 weeks of age does not induce efficiently SPA and when it does occur, an incubation period of several months is invariably seen (Rosadio, 1987).

The differences in the incubation period between the natural and the experimental model in new-born lambs could be due to different factors related to the virus and/or to the host itself.

First of all, the dose of the virus could have a direct and/or indirect effect over the length of the incubation period. It is difficult to have a precise figure regarding the dose of inoculum because no quantitative assays for JSRV are available at the moment. However, an approximate idea can be obtained by comparing the relative quantity of lung fluid used as inoculum (although the same amount of lung fluid collected from different animals can contain a different number of JSRV virions). Lambs are experimentally infected with the equivalent of 75 ml of lung fluid (75 ml of lung fluid ultracentrifuged and resuspended in 5 ml PBS; see chapter 2) while in nature it is reasonable to expect that sheep become infected with a few microlitres of lung fluid through aerosol or close contact between infected and uninfected sheep. Thus, the dose of virus used for the experimental reproduction of SPA is presumably several fold higher than the dose of virus transmitted in nature.

A “direct” effect of infection with a high virus dose would be the infection of a greater number of cells at the initial stages resulting in a faster progression of the disease.

An “indirect” effect of a high virus dose could be related to its effect on the host immune system. A high dose of virus in the inoculum could tolerate new-born lambs towards JSRV or impair their immune response and allow tumour to progress more rapidly. In adult sheep the presence of an antiviral immune response could explain the difficulty in experimentally reproducing the disease in adult sheep and the long incubation period in naturally acquired SPA.

This hypothesis is supported by the data accumulating on the MLV system. It has been known for a long time that the most efficient way to induce leukaemia with MuLV is through infection of new-born mice (Gross, 1951). Mice infected with MuLVs as new-borns generally show no virus-specific CTLs or circulating

antibodies (Oldstone *et al*, 1972; Chieco-Bianchi *et al*, 1974 and 1988; Green , 1984; Korostoff *et al*, 1990) and even when they raise antibodies to MuLV infection, this humoral response does not appear to influence the pathogenesis *in vivo* (Fan, 1994). In contrast, when adult mice are infected, an antiviral CTL response controls virus production and the appearance of disease (Chieco-Bianchi *et al*, 1988; Robertson *et al*, 1992).

Another possibility is that the differences in incubation period of SPA could be related to the cells target of JSRV. Epithelial lung cells could either express a JSRV receptor more abundantly or have an higher mitotic rate in new-born lambs. This last factor could facilitate JSRV infection and expression. It is well established that retroviral expression and continued viral expression is more efficient in dividing cells (Varmus *et al*, 1977; Humpries *et al*, 1981; Brown, 1990).

- **The ENTV system and its similarity with JSRV.**

As discussed in 1.1.10, many of the hallmarks of SPA have been found in ovine and caprine enzootic nasal tumour (ENT). These include (i) the experimental reproduction of the disease using cell-free filtrates of tumour homogenates and nasal exudates (Cohrs, 1953; De las Heras *et al*, 1995), (ii) the evidence in the tumour cells of type-A and type-D particles, (iii) the detection of particle-associated reverse transcriptase activity and (iv) capsid protein in the tumour that cross-reacts with MPMV (De las Heras *et al*, 1991b; Vitellozzi *et al*, 1993) and JSRV capsid protein (see Chapter 3).

Cousens *et al* (1996) amplified and sequenced a portion of ENTV-*gag* from sucrose gradient purified virions from three different nasal exudate samples (from three different farms) of ENT. Successful amplification was obtained employing the same primers used in the JSRV-PCR-*gag* (Chapter 4). Sequence analysis showed that ENTV sequences were 83 to 92% similar to JSRV sequences and 83 to 92% similar to *enJSRVs* sequences. The ENTV sequences lacked the *ScaI* site that is a marker for the exogenous JSRV but had two additional restriction sites, *AatII* and *PstI*, that

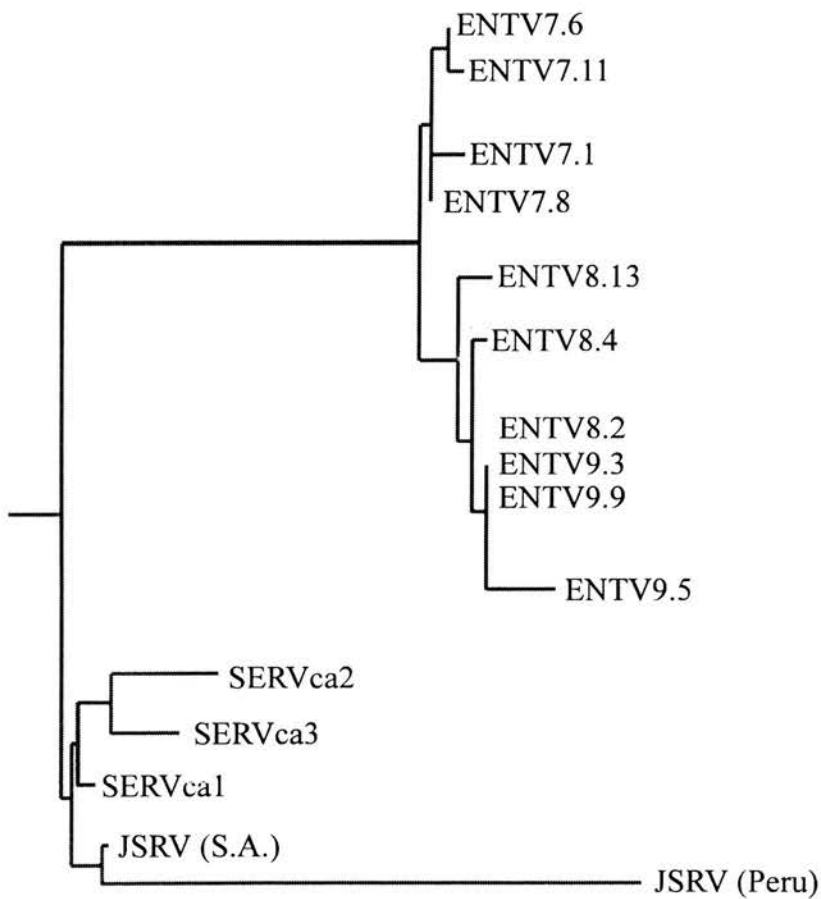


Fig. 7.1: **Phylogenetic tree constructed from portions of the *gag* gene of JSRV (JSRV[SA] and JSRV [Peru]) (York *et al*, 1992; Hecht *et al*, 1994), ENTV (Cousens *et al*, 1996) and *enJSRVs* (SERV) (Palmarini *et al*, 1996a). From Cousens *et al*, 1996.**

were absent in *enJSRVs*. These sites were present exclusively and consistently in ENTV from tumour tissues and exudates from ENT cases. Thus, ENTV is related to JSRV yet apparently distinct from it.

Phylogenetic analysis of these sequences showed that the JSRV and *enJSRV* sequences are more similar to each other than they are to the ENTV sequences (Fig. 7.1).

The consideration that these similar but distinct viruses are associated with similar but distinct epithelial tumours of the respiratory tract of small ruminants, support the hypothesis that these viruses are the primary cause of neoplasia.

7.2 Significance of SPA as a unique model of retroviral-induced lung cancer.

Retroviruses are an invaluable model system for understanding carcinogenesis and regulation of normal cell growth. Studies of spontaneous retrovirus-induced animal and human lymphomas have contributed immensely to our knowledge on cancer, yet relatively little information is available concerning retroviral causes and mechanisms of epithelial cell neoplasm which, in man, are much more frequent than lymphomas (Silverberg *et al*, 1990).

SPA represents a unique model of a naturally-occurring epithelial neoplasm which is associated aetiologically with a retrovirus. Interestingly, SPA shares certain characteristics with human bronchio-alveolar cell carcinoma (BAC) (Perk and Hod, 1982; Gazdar and Linnoila, 1988). Like SPA, BAC is a relatively well-differentiated tumour arising from type-II pneumocytes or Clara cells, the neoplastic tissues are preferentially localised at the periphery of the lungs and the lesions tend to be multifocal (Liebow, 1960). The multifocality of BAC has been explained either as the result of intrapulmonary lymphatic spread (metastasis) or a combination of aerosolization and aspiration (implantation) (Liebow, 1960).

The incidence of BAC is increasing dramatically in the US where it represents up to a quarter of primary lung carcinomas (Barsky *et al*, 1994a). The causes of BAC are unknown. However, several characteristics differentiate BAC from other primary

human lung tumours such as its relatively high incidence in females, its occurrence in non-smokers and in a relatively young age-group (Liebow, 1960; Ives *et al*, 1983). These features of BAC could suggest an aetiology distinct from other primary lung carcinomas.

A viral aetiology has been postulated for the similarity of BAC with SPA (Perk and Hod, 1982). Recently, multifocal BAC cases have been demonstrated to have a multiclonal origin (Barsky *et al*, 1994b); a multifocal-multiclonal neoplasia could be explained as viral-induced. The search for a retrovirus in BAC could well be fruitful. An immunohistochemical analysis of a group of human lung tumours including BAC, adenocarcinomas and epidermoid carcinomas has been recently performed (De las Heras, M., Palmarini, M., Hasleton, P., Minguijón, E., Gimenez-Mas, J.A., Dalziel, R.G., Egan, J., Larsson, E., and Sharp, J.M. *manuscript in preparation*) employing the rabbit antiserum to JSRV-CA described in Chapter 3 (Palmarini *et al*, 1995). Interestingly, a strong positive reaction has been obtained in the cytoplasm of the neoplastic cells in five out of the eight BAC cases examined. With the exception of a single adenocarcinoma all the other samples tested were negative including a control group of primary pulmonary hypertension cases.

These results can have different explanations:

- i) the rabbit antiserum cross-reacts aspecifically with antigens present in the BAC cells;
- ii) one of the HERVs is abundantly expressed in BAC tumour cells. HERV-K for example has 35% aminoacid identity with JSRV Gag;
- iii) a novel human JSRV-related virus is associated with BAC.

Of course, immunohistochemistry cannot provide conclusive results and more studies are much needed to explore these different scenarios. Preliminary indications can be obtained by western blotting (employing the same rabbit anti JSRV-CA serum) and RT-activity analysis of fresh frozen BAC tumour extracts. These tests would confirm the results obtained and represent a first step towards further studies at the molecular level.

Studies on the aetiology and pathogenesis of SPA could provide an invaluable model for BAC. The pathway of oncogenesis between the two tumours may be similar even if the hypothesis of a BAC-associated retrovirus should be proved wrong. For example the *int* oncogenes were identified solely by their association with MMTV-induced mammary tumours in mice and at least one of these oncogenes, *int-2*, is activated in some human cancers (Peters *et al*, 1989; Peters, 1991; Meyers and Dudley, 1992).

SPA might represent also a model to understand the biological relevance of human endogenous retroviruses. The major understanding of endogenous retroviruses have been obtained in inbred strains of mice or in selected lines of chickens which not necessarily reflects the biological function of endogenous retroviruses in humans which is, like sheep, an outbred animal species.

7.3 SPA as a model to investigate retrovirus-herpesvirus interaction.

Herpesviruses and retroviruses are notorious for causing a wide spectrum of disease with clinical courses ranging from acute to chronic, as well as persisting in the host in latent forms which are not accessible to the host immune system (Weiss *et al*, 1984; Wildy, 1986; Roizman *et al*, 1992).

Early studies on SPA showed an association with a herpesvirus (see 1.5; Mackay 1969a and 1969b; De Villiers *et al*, 1975; Martin *et al*, 1979) designated OHV-1. Subsequent studies discarded OHV-1 as an aetiological agent of SPA on the basis of epidemiological data and on the failure to experimentally reproduce SPA in sheep after inoculation of OHV-1 (Martin *et al*, 1976; Scott, 1984). Subsequently, the increasing evidence supporting JSRV as the aetiological agent of SPA has deterred investigators looking at the involvement of OHV-1 in the pathogenesis of SPA. However, it was intriguing that co-inoculation of JSRV and OHV-1 induced more extensive neoplastic lesions than the inoculation of JSRV alone (Martin *et al*, 1976). The strict association of retroviruses and herpesviruses with neoplastic diseases has generated considerable interest in studying the possibility that they could interact at

the cellular level in induction or progression of disease (Evermann *et al*, 1992). The occurrence of AIDS and the frequent concomitant infections with several herpesviruses, such as EBV, CMV, HSV-1, HHV-6 and more recently HHV-8 renewed interest in understanding possible synergistic actions of these two groups of viruses (Fauci *et al*, 1984; Salahuddin *et al*, 1986; Ablashi *et al*, 1987; Collier *et al*, 1989; Lusso *et al*, 1989; Watson, 1990; Schulz and Weiss, 1995). Synergism between herpesviruses and retroviruses can be attributed primarily to opportunistic infections due to immune compromising effects of one or both virus types. However, interaction at the cellular level also has been shown. Coinfection of cells with human CMV and HIV, for example, may augment HIV gene expression and virus production (Ho *et al*, 1990) and similarly, the infection of primary CD4⁺ and CD8⁺ T lymphocytes by EBV enhances HIV expression (Guan *et al*, 1996).

The interaction of CMV with HIV has been shown recently to be linked to the expression of a CMV-associated superantigen which enhances HIV-1 replication in CD4⁺ T cells bearing V β 12 TCRs (Dobrescu *et al*, 1995a and 1995b). Viral replication in targeted V β subsets may serve to promote a biologically relevant viral reservoir.

Immediate-early genes of HSV-1 are capable of transactivating HIV-LTR linked to reporter genes in cultured cells (Ostrove *et al*, 1987). HHV-6, which is predominantly a CD4⁺ T-lymphotropic virus (Lusso *et al*, 1988), also is capable of transactivating the HIV-1 LTR and productively coinfecting individual human T cells with HIV-1, causing an accelerated cytopathicity (Lusso *et al*, 1989).

Other well established examples of retrovirus-herpesvirus interactions have been shown once again with avian viruses. Lymphoid leukemia (LL) and Marek's disease (MD) are two distinct diseases of poultry caused by two different viruses. LL is a neoplasia of B lymphocytes caused by a retrovirus, the avian leukosis virus (ALV) (Purchase and Payne, 1984). MD is characterised by peripheral nerve demyelination and the formation of T lymphomas and it is caused by a herpesvirus, Marek's disease virus (MDV) (Payne, 1982). Coinfection of chicken with both ALV and MDV alters the disease processes associated with the individual viruses. The incidence of

Marek's disease was increased when chickens infected with pathogenic strains of MDV were also infected with ALV (Frankel and Groupe, 1971; Jakovleva and Maruzenko, 1979). Conversely, a vaccine strain of MDV enhanced the development of LL in chickens experimentally infected with ALV (Bacon *et al*, 1989). In the latter case an increase in the numbers of tumours per chicken as well as the frequency of metastatic tumours was observed. The non-pathogenic strain of MDV has been detected in ALV-transformed bursal B cells but not in normal B cells (Fynan *et al*, 1992) after experimental coinfection with the two viruses. It is possible that portions of the MDV genome transactivate the ALV LTR in the same way shown between MDV and Rous sarcoma virus (Banders and Coussens, 1994). Such a mechanism of transactivation could either enhance the ALV titre or the transcription of oncogenes under the control of the proviral promoter.

SPA therefore could serve as an animal model to elucidate the nature and potential effects of direct herpesvirus-retrovirus. This may have some implications in human medicine e.g. to investigate the pathogenesis of some HIV-associated herpesvirus infections.

7.4 Future priorities of SPA research.

In these last years there has been a significant improvement in the understanding of the biology of JSRV. JSRV has been completely sequenced (York *et al*, 1992), the sites of viral replication have been established (Palmarini *et al*, 1995 and 1996b) and the exogenous transmissible form of JSRV has been differentiated from the transcriptionally active *enJSRVs* (Bai *et al*, 1996; Palmarini *et al*, 1996a).

Investigators working on SPA now have the possibility to design experiments which will be able to define the aetio-pathogenesis of this important animal model.

SPA research can be arbitrarily divided into three main areas:

- i) studies on JSRV itself;
- ii) mechanisms of oncogenesis;
- iii) virus-host interaction:

- immune response towards JSRV virus;
- influence of *enJSRVs* on the pathogenesis of SPA.

i) **Studies on JSRV.**

One of the main obstacles to developing the SPA model has been the lack of an *in vitro* system for JSRV .

One way to prove the aetiology of SPA is to construct an infectious molecular clone (IMC) and experimentally reproduce the disease in neonatal lambs by direct inoculation of the IMC. Several approaches can be attempted to obtain an IMC.

One possibility is to derive an IMC from a genomic library constructed from the JS-7 cell line which derived from an SPA tumour (Jassim, 1988). Direct inoculation of JS-7 cells in young lambs, both at low and at high passage, reproduced SPA (Jassim, 1988). The cell line has been karyotyped and by inoculating lambs of the opposite sex, the tumours that developed were demonstrated not to have arisen from transplantation of the cell line (Jassim, 1988). The cell line, therefore, carries an infectious provirus and preliminary results seem to indicate that there is a single provirus integrated in this particular cell line (Palmarini M. and Sharp J.M., unpublished data). The use of a cell line instead of tumour could overcome the cloning of defective proviruses which can arise during retroviral replication as established in other systems such as ALV, HTLV and MuLV (Shields *et al*, 1978; Fung *et al*, 1981; Hiramatsu and Yoshikura, 1986; Robinson and Gagnon, 1986; Oshima *et al*, 1991).

The genomic library could be screened with exogenous-JSRV-specific oligonucleotides probes designed in the U3 of JSRV where major differences were shown between exogenous and endogenous LTRs (see chapter 5; Palmarini *et al*, 1996; Bai *et al*, 1996).

Obtaining a replication competent JSRV-IMC would be of great advantage for the SPA field. First, it would greatly assist research to identify the role of JSRV in the aetiology of SPA and second it would allow mapping of determinants of pathogenesis by construction of mutated clones.

A proviral clone also would allow confirmation of the only published complete sequence of JSRV (York *et al*, 1992). That sequence was obtained by sequencing clones of a cDNA library obtained from gradient purified lung secretions of a SPA sheep and by RT-PCR amplification of the missing portions of the JSRV genome. Of particular interest would be establishing whether the open reading frame (ORF-X) overlapping JSRV *pol* is a real gene or is an unusually long open reading that has arisen by chance. Sequencing of this region from isolates collected from unrelated geographical regions will help to address this issue.

ii) Mechanisms of oncogenesis.

The mechanisms of oncogenesis have to be completely elucidated. A priority towards this goal is to establish if SPA tumours are monoclonal/oligoclonal and establish if common integration sites occur between different tumours. If common integration sites exist in different tumours, this would be an indication of insertional mutagenesis. If instead integration sites are not localised to a few regions it may be speculated that other mechanisms are responsible for oncogenesis in SPA. Virus-cell flanking regions could be cloned either from a tumour genomic library or through a PCR approach (Sørensen *et al*, 1993) again utilising exogenous specific probes/primers in the U3. These flanking regions then could be used as probes in Southern blot analysis for comparison of pairs of tumour and non-tumour genomic DNAs digested with various restriction enzymes. Southern blots of non-tumour DNA would give an indication of the restriction map around the restriction site while blots of tumour DNA would show if JSRV insertion altered the mobility of the fragments.

iii) Virus-host interaction.

The interaction between JSRV and the host immune system is not known. Two basic questions have to be answered: are sheep tolerant towards JSRV or does JSRV induce an immune response?

The availability of the complete sequence of the JSRV genome will allow the generation of recombinant proteins which can be used to assess the humoral and

cellular immune response towards JSRV. The expression of JSRV recombinant proteins in eukaryotic instead of prokaryotic systems will avoid contamination of the protein preparations with LPS which interferes with tests designed to measure the cellular mediated immune response.

Simple ELISAs will establish whether or not sheep have antibodies towards JSRV. Experiments performed so far have included unaffected sheep as negative controls. However, sheep which do not show classical macro/microscopic SPA lesions are not necessarily free from infection with JSRV; unaffected sheep sera are therefore not sufficient as negative controls. It will be necessary to include control groups from sheep farmed in Australia or Iceland (where SPA has never been detected or it has been eradicated from more than 40 years) to enable correct interpretation of the results obtained.

Cellular mediated immunity could be investigated using the same recombinants in lymphoproliferative and cytokine (e.g. γ -interferon) assays. CTL responses would be more difficult to assess because CD8⁺ cells recognise autologous MHC-I (Male *et al*, 1987) and there is little information available about MHC haplotypes in sheep.

The sites of viral replication in the early stages of the disease also have to be established as, to date, only sheep with SPA at the terminal stages of the disease have been investigated (Palmarini *et al*, 1996a and 1996b). Analysis of viral integration and expression in experimentally infected lambs in the very first days post-infection could address this issue.

Another aspect that could elucidate the interaction between JSRV and its host is to further investigate the expression of *enJSRVs* for their possible influence on the pathogenesis of SPA. In particular, it should be investigated when and where *enJSRVs* are expressed during ontogeny.

The knowledge of humoral and cellular immune responses towards JSRV, the sites of viral replication and a better characterisation of *enJSRVs* expression will greatly advance our understanding on the interaction between JSRV and its host.

7.5 Conclusions

Sheep pulmonary adenomatosis is a unique model of a contagious epithelial neoplasm in an outbred animal species.

The results obtained during the course of this project have provided compelling lines of evidence which specifically associate jaagsiekte sheep retrovirus with SPA. The most plausible theory to explain this absolute association is that JSRV is 'the' aetiological agent of SPA or that it is at least primarily involved in the aetio-pathogenesis of the disease, perhaps as a virus helper of an as yet, uncharacterised acutely transforming retrovirus.

Several key aspects of the pathogenesis of SPA, such as the mechanisms of oncogenesis and the immune response towards JSRV, have still to be elucidated. However the improvements in the understanding of the biology of JSRV made in these last three years makes possible to design experiments to address these questions in the immediate future.

7.6 References

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Appendix I. Commonly used buffers

| | |
|-------------------------------------|--|
| Laemmli electrode buffer | 25 mM Tris, 192 mM glycine, 3.5 mM SDS, pH 8.3 |
| Laemmli sample buffer [X2] | 0.5 M Tris/HCl pH 6.8, 4% SDS, 8% β -mercaptoethanol, 20% [w/v] sucrose, 0.002% [w/v] bromophenol blue |
| L-broth | 10g tryptone, 5g yeast extract, 5g NaCl per l |
| L-agar | L-broth containing 15g agar per l |
| MOPS [10X] | 0.2M MOPS 3-[N-Morpholino] propanesulphonic acid , 50 mM sodium acetate and 10 mM EDTA, pH7 |
| PBS | 137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , pH 7.3 |
| Solution D | 4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M β -mercaptoethanol |
| SSC [20X] | 3M NaCl, 0.3M sodium citrate, pH 7 |
| TBE | 45 mM Tris/borate, 1 mM EDTA, pH 7.3 |
| TBS | 100 mM NaCl, 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 |
| TE | 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 |
| TNE | 100 mM NaCl, 10 mM Tris, 1 mM EDTA |
| Tris/glycine blotting buffer | 25mM Tris, 192 mM glycine, 20% [v/v] methanol, pH 8.3 |
| Washing fluid B | PBS, 0.5% (w/v)Tween 20, 1mM EDTA, 350 mM NaCl |

Appendix II. Oligonucleotides used in this project.

| Primers | Position* | Sequence (5'-3') |
|---------|-----------|-------------------------------------|
| P1 | 1598-1620 | GCTGCTTTRAGACCTTATCGAAA |
| P2 | 1826-1806 | ATACTGCAGCYCGATGGCCAG |
| P3960 | 1726-1747 | GGCTTGCTGCTGGAAAAGTACT |
| P4030 | 1726-1747 | GGCTTGCTGCTGGAAAAGTAAT |
| P4749 | 1700-1726 | GCATTACAAGGAAAAAGCATAAAAGGA |
| P3 | 7179-7193 | CTGCGGGGGACGAC |
| P4 | 111-125 | CTGCCGCGACCAGCA |
| P5 | 7210-7224 | TGGGAGCTCTTTGGCAAAGCC |
| P6 | 7361-7385 | CACCGGATTTTACACAATCACCGG |
| P7 | 7316-7350 | GGCACTGCTTCACAGAAATATCAGGAAATCTGATT |
| P8 | 7280-7313 | TTTTTAAAAG CTCTTAAGGCTCGGATGTTTGCTT |
| P9 | 7316-7338 | TGATATTTCTGTGAAGCAGTGCC |
| PA | 1726-1747 | GGCTTGCTGCTGGAAAAGTACT |

*= position referred to the JSRV sequence (York *et al*, 1992; GenBank accession # M80216).

Appendix 3. Papers published during the course of this thesis.

Palmarini, M., Dewar, P., De las Heras, M., Inglis, N.F., Dalziel, R.G. and Sharp, J.M. (1995). Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus. *Journal of General Virology* 76, 2731-2737.

Palmarini, M., Cousens, C., Dalziel, R.G., Bai, J., Stedman, K., DeMartini, J.C. and Sharp, J.M. (1996a). The exogenous form of Jaagsiekte retrovirus is specifically associated with a contagious lung cancer of sheep. *Journal of Virology* 70, 1618-1623.

Palmarini, M., Holland, M.J., Cousens, C., Dalziel, R.G., and Sharp, J. M. (1996b). Jaagsiekte retrovirus establishes a disseminated infection of the lymphoid tissues of sheep affected by pulmonary adenomatosis. *Journal of General Virology* 77, 2991-2998.

Cousens, C., Minguijon, E., Garcia, M., Ferrer, L.M., Daiziel, R.G., Palmarini, M., De las Heras, M., and Sharp, J.M. (1996). PCR-based detection and partial characterization of a retrovirus associated with contagious intranasal tumours of sheep and goats. *Journal of Virology* 70, 7580-7583.

Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus

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Sheep pulmonary adenomatosis (SPA) is a naturally occurring contagious lung tumour of sheep which has been associated aetiologically with a type D- and B-related retrovirus (Jaagsiekte retrovirus; JSRV). To improve understanding of the aetio-pathogenesis of SPA, the distribution and the sites of JSRV replication in sheep with naturally or experimentally induced SPA or in unaffected controls were identified. New immuno-

logical reagents were produced and a blocking enzyme-linked immunosorbent assay (B-ELISA) and an immunohistochemical technique for the detection of JSRV major capsid protein at the tissue and cellular levels were developed. JSRV was detected only in the respiratory tract of sheep affected by pulmonary adenomatosis and specifically in the transformed epithelial cells of the alveoli of SPA-affected sheep.

Introduction

Retroviruses related to the type D viruses of primates have been demonstrated in the house shrew (Tsutsui *et al.*, 1985; Aoyama *et al.*, 1988), sheep (Sharp & Herring, 1983; He *et al.*, 1992; De las Heras *et al.*, 1993), cattle (York *et al.*, 1989) and goats (De las Heras *et al.*, 1991). Of these type D viruses, only the simian retroviruses and the small ruminant retroviruses have been associated with any disease (Marx & Lowenstine, 1987; Sharp, 1987). The small ruminant viruses have been associated with three contagious tumours, sheep pulmonary adenomatosis (syn. SPA, jaagsiekte, ovine pulmonary carcinoma) (Sharp & Angus, 1990), enzootic nasal tumour of sheep (De las Heras *et al.*, 1993) and enzootic nasal tumour of goats (De las Heras *et al.*, 1991), all of which arise from secretory epithelial cells in the respiratory tract.

Each of these tumours has been shown consistently to contain a retrovirus with morphological, biochemical and immunological properties similar to type D retroviruses and cell-free tumour extracts can be used to reproduce these diseases experimentally (Cohrs, 1953; Martin *et al.*, 1976; Verwoerd *et al.*, 1980; Sharp *et al.*, 1983; DeMartini *et al.*, 1988; De las Heras *et al.*, 1995).

Further, the incubation period of experimentally induced sheep pulmonary adenomatosis (SPA) can be reduced to a few weeks or days (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983) and has been shown to be inversely related to the reverse transcriptase activity in the inoculum (Verwoerd *et al.*, 1985). Although several lines of evidence implicate a retrovirus, known as Jaagsiekte retrovirus (JSRV) in the aetiology of SPA (reviewed by Sharp, 1987), obtaining definitive proof by classical approaches has been impeded by the absence of a cell culture system to propagate JSRV and a lack of reagents and techniques to undertake studies on the pathogenesis of the disease. Recently, one strain of JSRV has been cloned and the full-length genomic sequence determined (York *et al.*, 1991, 1992). This virus exhibits the typical genomic organization of a replication-competent type D retrovirus with LTR, *gag*, *pro*, *pol* and *env* regions; there are no apparent sequences commonly associated with transformation (York *et al.*, 1992). Southern hybridization has revealed that numerous related sequences are present in the genomes of both unaffected sheep and goats (York *et al.*, 1992; Hecht *et al.*, 1994), which raises the question of whether JSRV is involved in the aetiology of SPA or is an endogenous homologue reactivated during the neoplastic process. The availability of one sequence of JSRV permitted new approaches in the investigation of the aetio-pathogenesis of SPA. Studies were undertaken, therefore, to examine the distribution and sites of replication of JSRV in SPA-affected and unaffected

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sheep to better define the role of type D retroviruses in the aetiology of SPA.

Methods

Subcloning and expression of clone Js382. Standard molecular biology procedures were used as described by Sambrook *et al.* (1989) and will not be reported in detail. Plasmid pBluescript-Js382 containing a part of the JSRV *gag* gene (bases 953 to 3030 of the nucleotide sequence published by York *et al.*, 1992) was a gift from G. Quérat (Marseille, France). Briefly, the insert fragment was excised by *EcoRI*, subcloned into plasmids pMS1S (Sherf *et al.*, 1990; supplied by M. Shreiber) and pGEX1 λ T (Pharmacia) and expressed in *E. coli* host strain NM522 as β -galactosidase (β gal-CA, plasmid pMCA) and glutathione *S*-transferase (GST-CA, plasmid pGCA) fusion proteins respectively.

Confirmation that the *gag* gene was in the correct reading frame was obtained by sequencing across the vector-insert junction, as well as testing clones for production of β -galactosidase and GST fusion proteins of the appropriate size by Western blotting (immunoblot) analysis with a goat antiserum to Mason-Pfizer monkey virus major capsid protein (MPMV-CA) (ref. 75S-148, National Cancer Institute Repository) (Sharp & Herring, 1983).

Transformed bacteria were grown and induced with isopropyl β -D-thiogalactopyranoside (IPTG) for the expression of recombinant proteins. Bacteria were pelleted (5000 *g* for 10 min) and resuspended in 20 ml TE (10 mM-Tris pH 7.5, 1 mM-EDTA). Phenylmethylsulphonyl fluoride (2 mM) was added before lysing the cell suspension in a French press at 1500 p.s.i. (10.35 MPa). The lysate obtained was sonicated and clarified at 100 000 *g* at 4 °C for 10 min.

β gal-CA fusion protein was purified by affinity chromatography using a 4 ml column of aminobenzyl 1-thio- β -galactopyranoside (ABTG) agarose (Sigma) as previously described (Cameron *et al.*, 1994). GST-CA was purified by affinity chromatography using a 4 ml column of glutathione-Sepharose (Pharmacia) as recommended by the manufacturers.

The yield of soluble β gal-CA was approximately 9 mg/l of bacterial culture at about 75% purity as estimated by SDS-PAGE. GST-CA could not be eluted with free glutathione from the Sepharose beads as recommended by the manufacturers and was therefore further used coupled to the beads to immunize rabbits.

Production of rabbit polyclonal antiserum to JSRV-CA. A specific rabbit antiserum to JSRV-CA was prepared by immunizing rabbits with 500 μ g β gal-CA combined with Freund's incomplete adjuvant. After 15 days the rabbit was boosted with 500 μ g of GST-CA bound to the glutathione-Sepharose beads. A third injection of GST-CA (500 μ g) was given after 4 weeks and the rabbit was bled 15 days after the last injection.

To avoid non-specific reactions in the B-ELISA and in the immunohistochemical study, the antiserum obtained was absorbed overnight at 4 °C with a lysate of IPTG-induced NM522(pMS1S) cells. The serum was then centrifuged at 100 000 *g* for 30 min to pellet any bacterial debris, aliquoted and stored at -20 °C until used.

By Western blotting the rabbit antiserum recognized the two recombinant proteins β gal-CA and GST-CA, as well as the native 25 kDa CA in JSRV. There was no reaction with any other protein in sucrose gradient purified JSRV (data not shown).

Sources and preparation of samples. In this study, SPA sheep were defined as animals which showed typical clinical signs, particularly the production of an abundant sero-mucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head, and confirmed by macroscopic and histological examination of the lungs.

To determine the best conditions for the B-ELISA and to assess its specificity and its inter- and intra-assay variability the following samples were employed: (a) two different pools of lung fluid (LFP#1 and 2) collected from several SPA sheep (in these samples the presence of JSRV was confirmed by Western blotting); (b) sucrose gradient purified maedi-visna virus (MVV); (c) a lysate of NM522(pMS1S) cells induced with IPTG to provide a β -galactosidase control; (d) a lysate of NM522(pGEX1 λ T) cells induced with IPTG to provide a GST control.

LFP#1 was filtered through a double layer of gauze and clarified by centrifugation at 100 000 *g* for 1 h at 4 °C. The resultant supernatant was aliquoted and stored at -70 °C until use.

JSRV was extracted from LFP#2 as described by Herring *et al.* (1983). Briefly, LFP#2 was filtered, clarified and the supernatant was then centrifuged at 100 000 *g* through a double layer of glycerol (25/50%, v/v) for 1 h at 4 °C. An aliquot of the supernatant was stored at -70 °C before and after the ultracentrifugation step. The resultant pellet was resuspended in TNE buffer (100 mM-NaCl, 10 mM-Tris, 1 mM-EDTA) and further purified by isopycnic centrifugation on 20 to 55% (w/w) sucrose gradients. The gradient was fractionated and each 0.5 ml fraction was resuspended in 4.5 ml TNE buffer, centrifuged at 100 000 *g* for 1 h at 4 °C and the resultant pellet resuspended in 100 μ l TNE.

For analysis of the distribution of JSRV in affected and unaffected animals, samples were collected from: (a) nine sheep naturally affected by SPA; (b) 16 lambs, experimentally infected as previously described (Sharp *et al.*, 1983); (c) 16 age and breed-matched controls.

Samples of lung, lung tumour, mediastinal and retropharyngeal lymph nodes, thymus, tonsils, Peyer's patches, spleen, bone marrow and kidney were collected during the post-mortem examination or immediately before the sheep were killed (lung fluid and heparinized blood samples). Lung fluid samples were filtered, clarified at 100 000 *g* for 1 h at 4 °C and the resultant supernatants were stored at -70 °C. Plasma samples (8 ml per sample) were further centrifuged at 100 000 *g* for 1 h at 4 °C and the pellets resuspended in 65 μ l of TNE before storing at -70 °C until use. Leukocytes were obtained from blood samples in which the red cells had been lysed. Each leukocyte sample (7×10^7 cells) was diluted in TNE, frozen/thawed three times, and clarified and centrifuged as described above. After the ultracentrifugation step, pellets were resuspended in 65 μ l of TNE and stored at -70 °C.

Tissues samples were homogenized [10% (w/v) suspension in TNE], filtered, clarified and centrifuged as above. All tissue samples were concentrated 400 times except tonsils which were concentrated only 100 times due to the small size of this organ. Tissues extracts were kept at -70 °C until further use.

Blocking enzyme-linked immunosorbent assay (B-ELISA). The SPA B-ELISA (Engvall & Perlmann, 1971) depends on the presence of JSRV in the test samples to inhibit the binding between purified β gal-CA and the rabbit antiserum to JSRV-CA.

Equal volumes of the test sample and washing fluid B [WFB; PBS, 0.5% (w/v), Tween 20, 1 mM-EDTA and 350 mM-NaCl] were reacted at room temperature for 1 h in order to disrupt the envelope of JSRV and release the capsid of the virions. WFB was also used as washing buffer for the ELISA plates and as dilution buffer for the reagents of the reaction. The optimal concentrations of β gal-CA and the rabbit antiserum were determined before use. Microtitre plates were coated with 100 μ l of purified β gal-CA (1.5 μ g/ml), diluted in carbonate buffer (pH 9.6), by absorbing overnight at 4 °C in a humid chamber. After incubation, the plates were washed with WFB and blocked by incubation for 1 h at 37 °C with a 1% (w/v) solution of bovine serum albumin in PBS.

The antiserum and test samples were reacted first in a V-bottom 'transfer' plate (65 μ l of 1:800 dilution of rabbit antiserum to JSRV-CA + 65 μ l of test samples pre-treated with WFB) for 1 h at 37 °C.

Thereafter, 100 µl of the resulting mixture (rabbit serum + sample) was transferred to the pre-coated ELISA plates, which had been washed three times with WFB.

The ELISA plates were incubated at 37 °C for 1 h and, after washing, 100 µl of pig antiserum to rabbit IgG conjugated to horse-radish peroxidase (DAKO; 1:1000 in WFB) was added to each well. After incubation at 37 °C for 1 h, the plates were washed again with WFB and 100 µl of substrate (*o*-phenyldiamine; Sigma) + 0.05% H₂O₂ (30%, v/v) was added to each well. The colour development was stopped with 2.5 M-H₂SO₄ and the plates read using a Dynatech MR5000 apparatus at 490 nm.

Samples were tested in duplicate in each test. Controls included wells with no inhibitor (0% blocking), and wells with neither inhibitor nor serum (100% blocking), the missing component being replaced with diluent (WFB).

Results are expressed as a percentage blocking value calculated using the following formula

$$\% \text{ blocking value} = [(OD_0 - OD_{\text{test}}) / (OD_0 - OD_{100})] \times 100$$

where OD₀ is the OD at 0% blocking control, OD₁₀₀ is the OD at 100% blocking control and OD_{test} is the OD of the test sample.

The intra-assay standard deviation was evaluated testing LFP#1 across the ELISA plate in five different plates and calculating the mean between the standard deviations obtained in each plate. The inter-assay standard deviation was calculated assaying LFP#1 in ten independent tests performed on different days. LFP#1 was located in the same wells on the ELISA plate on each occasion.

Western blotting. Western blotting was performed on the purified βgal-CA and GST-CA and on LFP#1 sucrose gradient purified fractions employing goat antiserum to MPMV-CA and the rabbit antiserum to JSRV-CA as described previously (Sharp & Herring, 1983).

Immunohistochemistry. Tissue samples used in immunohistochemistry studies were collected during the post-mortem examination from four SPA-affected sheep (three experimentally infected lambs and one natural case of SPA) and three uninfected controls. The same tissues as collected for the B-ELISA were fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned at 4–6 µm. Selected sections were stained immunohistochemically using a commercial avidin-biotin peroxidase complex kit (Vectastain ABC kit; Vector Laboratories) as recommended by the manufacturers except that sections were treated additionally with 0.1% (w/v) trypsin in TBS for 30 min at 37 °C. The rabbit antiserum to JSRV-CA was used as primary antibody at the predetermined optimal dilution of 1:50. This serum was substituted with TBS to control for the endogenous peroxidase activity of the tissue or with rabbit pre-immunization serum to check for non-specific reactions. Carazzi's haematoxylin was used as a counterstain.

Results

Development and validation of the B-ELISA

A series of experiments was performed to demonstrate that the blocking values obtained in the B-ELISA were the result of the interaction between JSRV-CA and the rabbit antiserum produced in this study and not due to non-specific reactions.

The analysis of the 18 fractions of LFP#2 separated by isopycnic centrifugation showed that the greatest % blocking values corresponded to fractions having a buoyant density between 1.16 g/ml to 1.19 g/ml, with a peak at 1.18 g/ml which is the buoyant density of JSRV

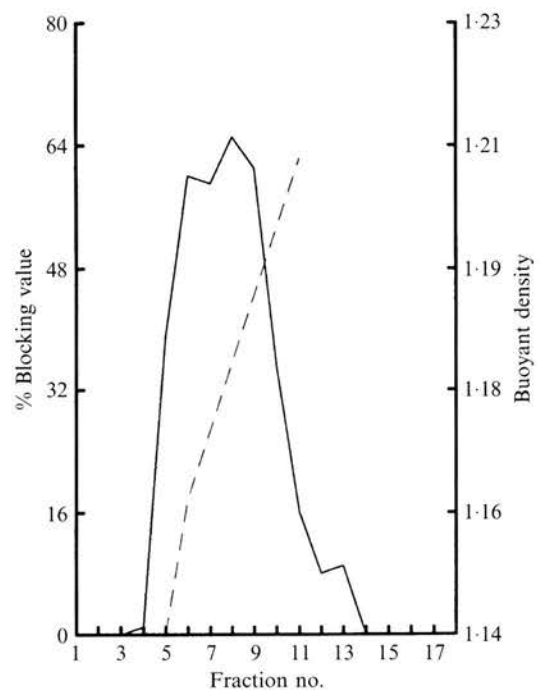


Fig. 1. Percentage blocking value of LFP#2 after isopycnic centrifugation in 20 to 55% (w/w) sucrose gradients. The continuous line shows the % blocking values; the broken line shows density (g/ml) after sucrose gradient centrifugation.

(Sharp & Herring, 1983) (Fig. 1). The same fractions analysed by Western blotting, employing a goat antiserum to MPMV-CA and the rabbit serum produced in this study showed a single polypeptide with an *M_r* of about 25000, the predicted *M_r* of JSRV-CA.

In LFP#2 the blocking value before ultra-centrifugation was 63% which was reduced to 10% for the post-centrifugation supernatant demonstrating that the blocking activity was particle-associated, and did not arise as a result of antibodies specific for JSRV-CA that might have been present in the lung fluid. In other experiments maedi-visna virus, another retrovirus of sheep which is occasionally found to coinfect SPA-affected animals (Rosadio *et al.*, 1988*a, b*), the β-galactosidase control and the GST control gave % blocking values less than 7.

These data demonstrate that the blocking value of the SPA B-ELISA depends on the presence in the test samples of the major capsid protein (CA) of JSRV.

The intra-assay standard deviation was ±1.6% while the inter-assay standard deviation was ±4.6%.

Distribution of JSRV viral particles in tissue extracts of SPA-affected and unaffected sheep

Following the successful development of the B-ELISA to detect JSRV-CA, this assay was used to examine the anatomical distribution of JSRV in sheep. The samples

Table 1. Results of the B-ELISA on tissues of SPA affected and unaffected sheep

| Sample | SPA + * | | SPA - † | |
|-----------------------------|------------------------------|----------------|------------------------------|-----|
| | % blocking value (\pm SE) | n^{\ddagger} | % blocking value (\pm SE) | n |
| Lung tumour/lung | 77.8 (\pm 4.8)§ | 13 | 17 (\pm 1.8) | 13 |
| Lung fluid | 68.4 (\pm 3.3) | 15 | — | — |
| Mediastinal lymph nodes | 15.2 (\pm 3.7) | 12 | 6.2 (\pm 2.1) | 8 |
| Retropharyngeal lymph nodes | 6.6 (\pm 1.4) | 8 | 7.0 (\pm 2.4) | 7 |
| Tonsils | 4.7 (\pm 2.3) | 9 | 3.7 (\pm 1.5) | 9 |
| Bone marrow | 40.2 (\pm 4.06) | 6 | 45.2 (\pm 5.7) | 6 |
| Thymus | 18.7 (\pm 3.8) | 7 | 13.1 (\pm 2.7) | 13 |
| Peyer's patches | 12.6 (\pm 1) | 5 | 7.8 (\pm 2.7) | 6 |
| Spleen | 18.7 (\pm 2.7) | 9 | 16.1 (\pm 3.9) | 8 |
| Kidney | 6.5 (\pm 2.9) | 8 | 8.3 (\pm 4.1) | 7 |
| Plasma | 10.8 (\pm 3) | 10 | 7.6 (\pm 3.1) | 5 |
| Leukocytes | 12.4 (\pm 2.2) | 10 | 13.4 (\pm 1.1) | 5 |

* SPA-affected animals.

† Unaffected controls.

‡ Number of samples tested.

§ Standard error of the mean.

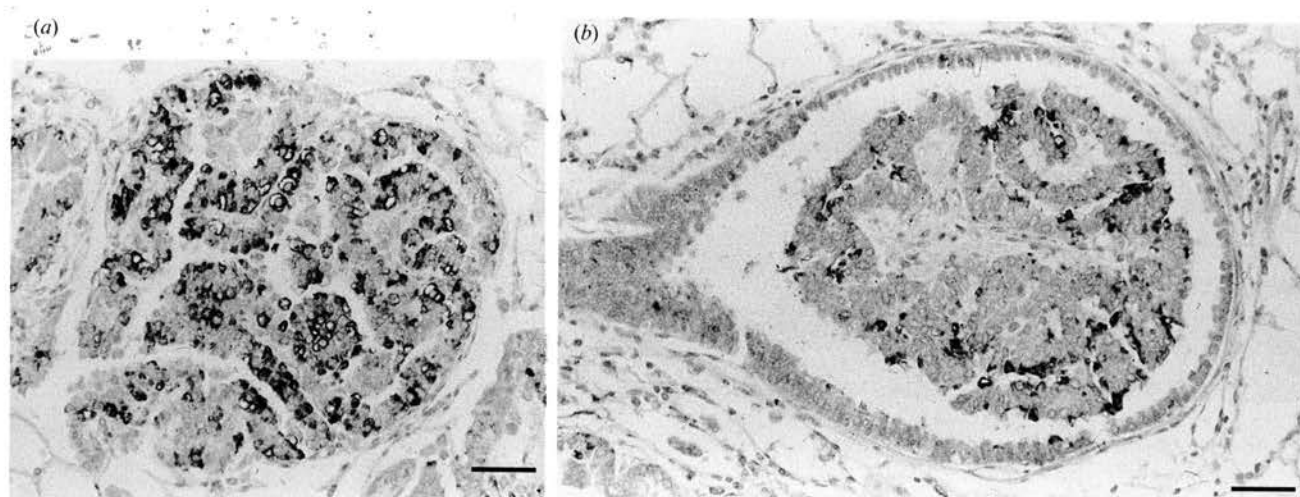


Fig. 2. (a, b) Immunohistochemistry of a lung section of a sheep affected by SPA (bars, 40 μ m; magnification 250 \times). The dark staining represents the positive reactions and is confined to the cytoplasm of the transformed cells of the adenoma. No staining is apparent in the extracellular airway spaces. A papillary projection of the tumour in the bronchiolus is visible in (b). The dark staining is confined to the neoplastic cells. No staining is observed in the interstitial cells or in the untransformed alveolar cells and bronchiolar cells.

tested were obtained by ultracentrifugation and therefore reflected the presence of JSRV viral particles. Statistical analysis of the differences between SPA-affected and unaffected animals was undertaken using Student's *t*-test. Results are summarized in Table 1.

Only tumour and lung samples showed a significant difference between affected and unaffected sheep ($P < 0.00001$). The mean % blocking value of tumour samples was 77.8 with values ranging from 41 to 93 while normal lung samples had a mean % blocking value of 17 with a range from 0 to 33.

A % blocking value of 36 was selected as the highest negative value calculated from the mean blocking value

of control lungs plus 2.5 standard deviations. When this value was used no false positive or false negative samples were observed.

No significant difference was observed between the mean blocking values obtained using lung tumour samples collected from natural or from experimentally induced cases of SPA. Lung fluid samples from SPA cases showed a high % blocking value (mean = 68.4) with values ranging from 49 to 92 but because healthy sheep do not produce collectable quantities of lung fluid no direct control for this sample could be obtained. The closest control for the lung fluid samples was broncho-alveolar lavages from unaffected sheep, in which

% blocking values were always under 18 (data not shown).

In all the other tissues examined there was no statistically significant difference between SPA-affected and unaffected sheep with the exception of a single mediastinal lymph node sample, collected from a natural case of SPA. This sample had a % blocking value of 53, markedly greater than the mean % blocking value of equivalent tissues in control animals (6.25).

Bone marrow samples showed a high mean blocking value in both affected and unaffected controls. The mean % blocking values were 40.2 for SPA sheep and 45.2 for the unaffected controls. These high % blocking values are probably an artefact due to the nature of the sample after processing. The preparation of this tissue as described in Methods resulted in a viscous pellet which therefore could have blocked the reaction non-specifically. Further dilutions or sucrose gradient purified samples resulted in the almost complete loss of blocking activity and no specific reaction in this tissue was found by immunohistochemistry (see below) or Western blotting.

Cellular localization of JSRV-CA

Immunohistochemical analysis showed that JSRV major capsid protein was detected in the cytoplasm of recognizable alveolar neoplastic cells in all four SPA lungs examined (Fig. 2*a, b*). There was no staining of the epithelial non-transformed cells nor of the interstitial cells. Specific staining in the extracellular airway spaces was observed very rarely. No specific staining was detected in any other tissue.

Discussion

The results of this study using immunological techniques have shown that JSRV virions and CA were detected only in the respiratory tract of sheep affected by SPA. These findings have confirmed earlier reports describing type D retrovirus antigens or reverse transcriptase activity in respiratory tissues of SPA sheep (Perk *et al.*, 1974; Martin *et al.*, 1976; Herring *et al.*, 1983; Sharp & Herring, 1983; Verwoerd *et al.*, 1983; Rosadio *et al.*, 1988*a*) and extended these observations by demonstrating that JSRV was not detected in 172 samples from eight other tissues from 41 sheep, although viral expression, below the detection limit of the B-ELISA, may be occurring in other tissues.

Immunohistochemistry supported the above conclusion and further indicated that JSRV replication occurred primarily in the transformed epithelial cells in the pulmonary alveoli of SPA sheep. A notable exception was the positive result obtained with a single lymph node

from a field case of SPA that probably arose as a consequence of metastatic events, which are known to occur in a proportion of cases (Nobel *et al.*, 1969). Although immunohistochemical studies employing a heterologous antiserum to MPMV-CA detected putative JSRV antigens in the alveolar lumina, rather than intracellularly (Payne *et al.*, 1986), and therefore conflict with those reported here, the observations of the present communication, obtained with an homologous antiserum, are in agreement with earlier ultrastructural studies describing intracytoplasmic type A particles in the epithelial tumour cells and, associated with these cells, a few extracellular particles with typical type D morphology (Perk *et al.*, 1971; Sharp *et al.*, 1983; Payne *et al.*, 1983).

The above findings point to the epithelial tumour cells in the alveoli as the major site of translation and assembly of JSRV and highlight the strict association between JSRV and the tumour. These findings, therefore, provide further support for the notion that JSRV is the aetiological agent of SPA. Although most type D retroviruses appear not to be pathogenic, a simian type D retrovirus (SRV 2) has been associated aetiologically with retroperitoneal fibromatosis in macaques (Bryant *et al.*, 1986) and the other sheep and goat type D-related viruses have been implicated in epithelial tumours (De las Heras *et al.*, 1991, 1993, 1995; Vitellozzi *et al.*, 1993). Thus, in contrast to other retrovirus models involving leukaemogenic viruses, these sheep and goat retroviruses may represent unique models of oncogenic retroviruses associated with the development of naturally occurring epithelial carcinomas (Cremer & Gruber, 1992).

Whilst the circumstantial evidence implicating JSRV in neoplasia is compelling, the existing information does not rule out other possibilities in which it may, or may not, be involved in the neoplastic processes. JSRV may be acting only as a helper for some other replication-defective, acutely transforming retrovirus or result from reactivation of endogenous viral sequences as a consequence of neoplasia, as seen with other endogenous retroviruses (Weiss *et al.*, 1985). The presence of up to 15 to 20 copies per genome of sequences related to the *gag*, *pol* and *env* genes of JSRV (York *et al.*, 1992; Hecht *et al.*, 1994) introduces major complications, particularly as endogenous retroviruses have been demonstrated in mice, chickens and cats to be involved in recombination events leading to the generation of oncogenic retroviruses (Stoye *et al.*, 1991; Golovkina *et al.*, 1994; McDougall *et al.*, 1994; Bai *et al.*, 1995). The occurrence of such events in SPA could be indicated by the apparent lack of serum antibodies to JSRV-CA in affected sheep or their unaffected flockmates (Sharp & Herring, 1983). It is clear, therefore, that further studies will be required to determine the role of JSRV in the aetiology of SPA and

the relationship between the exogenous and endogenous viruses.

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The Exogenous Form of Jaagsiekte Retrovirus Is Specifically Associated with a Contagious Lung Cancer of Sheep

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Sheep pulmonary adenomatosis (SPA) (ovine pulmonary carcinoma) is a transmissible lung cancer of sheep that has been associated etiologically with a type D- and B-related retrovirus (jaagsiekte retrovirus [JSRV]). To date it has been impossible to cultivate JSRV *in vitro* and therefore to demonstrate the etiology of SPA by a classical approach. In addition, the presence of 15 to 20 copies of endogenous JSRV-related sequences (*enJSRV*) has hampered studies at the molecular level. The aim of this study was to investigate whether the expression of exogenous JSRV was specifically associated with neoplasia in SPA-affected animals. Initially, we found that *enJSRV*s were transcribed in a wide variety of normal sheep tissues. Then, by sequencing part of the *gag* gene of *enJSRV* we established a *ScaI* restriction site in *gag* as a molecular marker for the exogenous form of JSRV. Restriction enzyme digestion of PCR products obtained from the amplification of cDNA from a total of 65 tissues collected from SPA-affected and unaffected control sheep revealed that the exogenous form of JSRV was exclusively and consistently present in tumor tissues and lung secretions of the affected animals. In addition, exogenous JSRV provirus was detected only in DNA from SPA tumors and not from nontumor tissues of the same animals. This study has demonstrated clearly that the exogenous form of JSRV is specifically associated with SPA tumors.

Retroviruses are an invaluable model system for understanding carcinogenesis and the regulation of normal cell growth. Studies of spontaneous retrovirus-induced animal and human lymphomas have contributed immensely to our knowledge of the onset of cancer (5), yet relatively little information is available concerning retroviral causes and mechanisms of epithelial cell neoplasms which, in humans, are much more frequent than lymphomas (39).

Sheep pulmonary adenomatosis (SPA; also known as jaagsiekte and ovine pulmonary carcinoma) represents a unique model of a naturally occurring epithelial neoplasm which is associated etiologically with a retrovirus (6, 35). Interestingly, SPA shares certain characteristics with human bronchoalveolar cell carcinoma (13, 29), which is only weakly associated with smoking (10, 16, 21) and which has dramatically increased in prevalence since 1955 and now represents 24% of lung cancers (3).

Several lines of evidence implicate retroviral involvement in the etiology of SPA. SPA has been reproduced experimentally by inoculating sheep with cell-free lung secretions (lung fluid) or tumor extracts from SPA-affected animals (7, 24, 36, 42). These materials have been shown consistently to contain a retrovirus with morphological, biochemical, and immunological properties similar to type D and B retroviruses (20, 30, 32, 38). This virus, known as jaagsiekte retrovirus (JSRV; also known as SPA retrovirus and ovine pulmonary carcinoma retrovirus), exhibits the typical genomic organization of a replication-competent retrovirus with long terminal repeat (LTR), *gag*, *pro*, *pol*, and *env* regions and no apparent sequences commonly associated with transformation (43). Despite the clear

association between JSRV and pulmonary adenomatosis, classical approaches to demonstrate an etiological role for this virus have been hampered by the lack of a cell culture system to propagate JSRV.

Recently, the link between JSRV and SPA was strengthened further by the demonstration that lung and transformed pulmonary epithelial cells of SPA-affected sheep are major sites of virus replication. JSRV particles or antigens have never been detected in tissues of unaffected control sheep (27). In addition, the presence of 15 to 20 copies of endogenous JSRV-related sequences (*enJSRV*) in the normal sheep genome has hampered analysis at the molecular level (19, 43). No information is available about *enJSRV* sequences and their degree of similarity with the exogenous JSRV. Therefore, the possibility cannot be excluded that expression of the exogenous virus in neoplastic cells is the result of the reactivation of an *enJSRV* as a downstream event of the neoplasia.

The aim of this study was to investigate expression of JSRV in tumor tissues and whether it is specifically associated with neoplasia. Initially, the expression of JSRV-related sequences in tissues of SPA-affected and unaffected control sheep was determined by reverse transcription-PCR (RT-PCR). Differentiation between exogenous and endogenous RNA species was evaluated by sequencing and/or restriction enzyme digestion of PCR products obtained from viral pellets and tumors of SPA-affected sheep and tissues of unaffected sheep. Comparison between tumor and nontumor genomic DNA collected from the same SPA-affected animals was then performed in order to determine whether the exogenous proviral JSRV was detectable only in genomic DNA from tumor tissues.

MATERIALS AND METHODS

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Sources and preparation of samples. In this study, SPA-affected sheep were defined as animals which showed typical clinical signs, particularly the produc-

TABLE 1. Tissues examined by RT-PCR (*gag*) followed by *ScaI* digestion in SPA-affected and unaffected control sheep

| Tissue | SPA ^a | | SPA ^b | |
|------------------------|----------------------------------|---|----------------------------------|---|
| | PCR (no. tested/no. positive) | <i>ScaI</i> digestion (no. tested/no. sensitive) | PCR (no. tested/no. positive) | <i>ScaI</i> digestion (no. tested/no. sensitive) |
| Lung fluid | 16/16 | 16/16 | | |
| Tumor/lung | 5/5 | 5/5 | 4/4 | 4/0 |
| Kidney | 3/3 | 3/0 | 3/3 | 3/0 |
| Thymus | 2/2 | 2/0 | 3/3 | 3/0 |
| Bone marrow | 3/3 | 3/0 | 3/3 | 3/0 |
| Spleen | 2/2 | 2/0 | 3/3 | 3/0 |
| Mediastinal lymph node | 5/5 | 5/3 | 3/3 | 3/0 |
| Leukocytes | 5/5 | 5/0 | 5/5 | 5/0 |
| Total | 41/41 | 41/24 | 24/24 | 24/0 |

^a SPA-affected sheep.^b Unaffected control sheep.

tion of abundant seromucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head. Diagnosis was confirmed by macroscopic and histological examination of the lungs. Both naturally SPA-affected animals and lambs infected by intratracheal inoculation of concentrated lung fluid, as previously described (36), were used in this study. In addition, age- and breed-matched controls were employed.

A total of 16 lung fluid samples were collected during clinical examinations of SPA-affected sheep and processed as already described (38). Briefly, 5 to 10 ml of lung fluid was filtered through a double layer of gauze and clarified by centrifugation at $10,000 \times g$ for 1 h at 4°C. The supernatant was then centrifuged at $100,000 \times g$ through a double layer of glycerol (25 and 50% [vol/vol]) for 1 h at 4°C, and the resultant pellet was resuspended in 500 μ l of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% sarcosyl, 0.1 M β -mercaptoethanol) and stored at -20°C until use. Leukocytes were obtained from 10 blood samples in which the erythrocytes had been lysed. A total of 10^7 cells for each sample were resuspended in 500 μ l of solution D and stored at -20°C.

Tissues from five lung tumors, four normal lungs, eight mediastinal lymph nodes, five thymus glands, six bone marrow samples, five spleens, and six kidneys of both SPA-affected and unaffected sheep were collected at postmortem examination (Table 1) and snap-frozen in liquid nitrogen before storage at -70°C. Dog lung tissues were treated as the sheep tissues and used as negative controls. An equine dermis cell line and primary fetal lamb lung and kidney cells were employed as further controls in this study. Cells (10^7) were resuspended in 500 μ l of solution D and stored at -20°C.

RNA extraction. Total RNA from ultraspeed pellets of lung fluid (2.5 to 5.0 ml), sheep tissues, and dog lung tissues (0.2 to 0.5 g), leukocytes, equine dermis cells, and fetal lamb lung and kidney cells (10^7 cells) was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (4). Each RNA sample was treated with 10 U of DNase I, RNase free (Boehringer), as suggested by the manufacturer.

DNA extraction. High-molecular-weight genomic DNA was extracted by established procedures (34) from lung tumor and kidney samples from four of the five SPA-affected animals and from lung and kidney tissues from three of the four unaffected controls. DNA was also isolated from primary cultures of normal sheep skin fibroblasts for analysis of the LTRs (see below). Briefly, pulverized tissue or cell pellets were lysed in TES buffer (10 mM Tris, 0.1 M EDTA, 0.5% sodium dodecyl sulfate [pH 8]) followed by digestion with RNase A (Sigma; 20 μ g/ml) for an hour at 37°C with gentle mixing, then 100 μ l (20 μ g/ml) of proteinase K was added and incubated at 60°C overnight. Separate phenol and chloroform-isoamyl alcohol (24:1) extractions were followed by isopropanol precipitation. High-molecular-weight DNA was harvested by spooling it onto glass pipettes and was washed twice with 70% ethanol on ice, dried, resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]), and stored at 4°C.

RT-PCR (*gag*). Synthesis of cDNA was carried out in 25- μ l reaction volumes as follows. Total RNA (2 μ g from tissues, leukocytes, and cell culture samples or total RNA obtained from ultraspeed pellets of 5 to 10 ml of lung fluid) was diluted in 13.5 μ l of diethyl pyrocarbonate-treated distilled water. RNA was denatured at 65°C for 10 min and then cooled on ice. One microliter (40 U) of rRNasin (Promega)-2 μ l of 25 mM each deoxynucleoside triphosphate (dNTP)-2.5 μ l of 100 mM dithiothreitol-2.5 μ l (A_{260} 62.5 U/ml [Boehringer]) of random hexamers-2.5 μ l of $10 \times$ reaction buffer (500 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$)-1 μ l of StrataScript reverse transcriptase (50 U; Stratagene) was added. The addition of the reverse transcriptase was omitted in control samples to check for DNA contamination of the RNA preparations. The reaction mixture was incubated at 37°C for 1 h, and then the enzyme was inactivated by incubation at 94°C for 3 min.

Amplification of the resultant cDNA was carried out by PCR. Primers were

designed on the basis of the complete sequence of JSRV (43) and the pCA1 sequence of *gag* (19). Primers P1 and P2 (Fig. 1A) spanned a 229-bp region internal to the *gag* gene (position 1598 to 1826 of JSRV) (43) (sense [P1], 5'-GCTGCTTTRAGACCTTATCGAAA-3'; antisense [P2], 5'-ATACTGCAG CYCGATGGCCAG-3'). Three microliters of cDNA was added to 47 μ l of $1 \times$ PCR buffer (1.25 U of *Taq* polymerase [Boehringer], 2.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 200 μ M each dNTP, 6.25 pmol of each primer). PCR cycles employed were 94°C for 1 min and 35 cycles of 94°C for 45 s, 57°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 2 min in a Perkin-Elmer GenAmp 2400 thermal cycler.

PCR was optimized for the detection of 1 to 10 copies of target DNA, using plasmid pJS382 (courtesy of G. Querat) containing part of the JSRV *gag* gene (nucleotide 953 to 3029) as template. Amplified products were detected by electrophoresing 25- μ l aliquots through a 2% agarose gel in $1 \times$ TBE buffer in the presence of 0.5 μ g of ethidium bromide per ml.

The products of five independent PCR-*gag* procedures with kidney cDNA from an unaffected sheep were pooled before being cloned in pGEM-T (Pro-

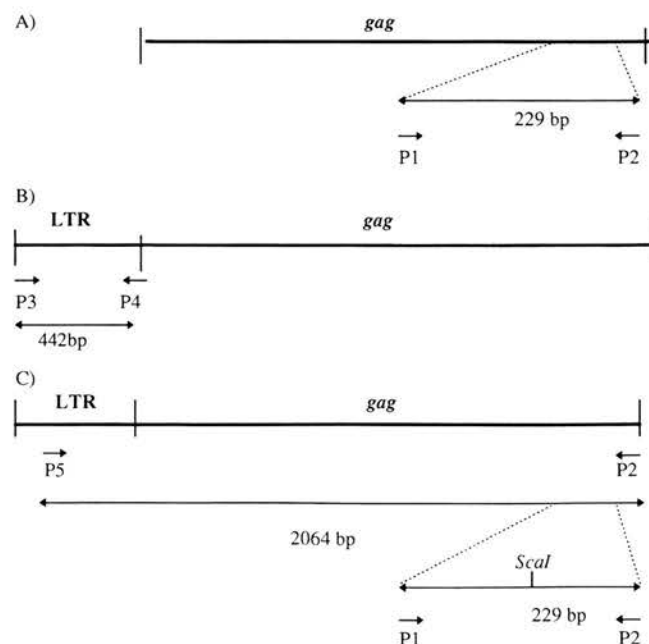


FIG. 1. PCR strategy and localization of primers employed in this study. (A) RT-PCR (*gag*), employing primers P1 and P2, amplified a product of 229 bp. (B) PCR for the amplification of endogenous LTRs employed primers P3 and P4 and generated products of 442 to 444 bp. (C) Heminested LTR-*gag* PCR. The first round of amplification used primer P5 (internal to the LTR) and *gag* primer P2. One microliter of this reaction mixture was used in the second round of amplification, which used primers P1 and P2.

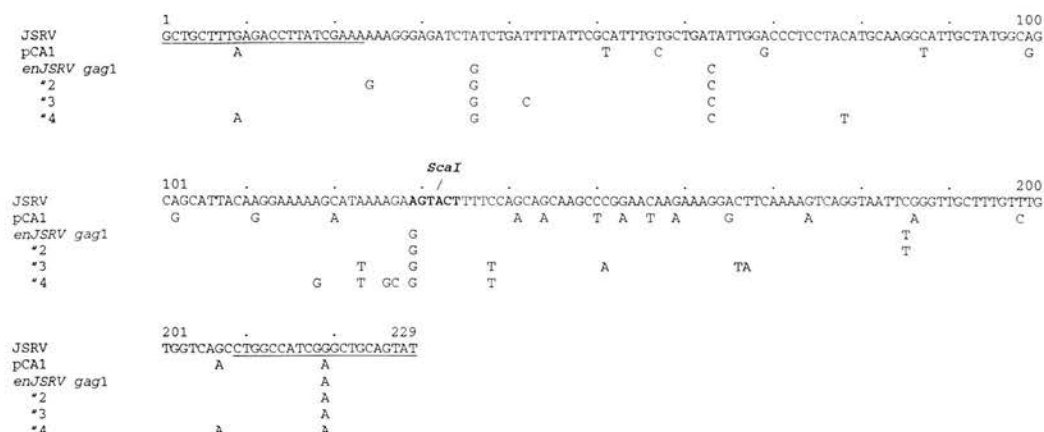


FIG. 2. Comparison of *enJSRV gag* sequences and exogenous pCA1 (19) with JSRV (43). Indicated are base differences with JSRV. Primers used are underlined, while the boldface bases indicate the exogenous-specific *ScaI* site.

mega), following the manufacturer's protocol. Seven clones were selected and sequenced with the T7 sequencing kit (Pharmacia), as recommended by the manufacturer.

Restriction enzyme digestion. Twenty-five microliters of the PCR-gag product was digested with 13 U of *ScaI* (Boehringer), employing the appropriate incubation buffer supplied by the manufacturer (PCR buffer was ignored), at 37°C for 2 to 4 h. The digestion products were analyzed alongside the corresponding mock-digested PCR products, by electrophoresis in a 2% agarose gel stained with ethidium bromide.

Amplification and sequencing of endogenous LTRs. One hundred nanograms of high-molecular-weight genomic DNA isolated from sheep skin fibroblasts was used for PCR amplification of endogenous LTRs (PCR-LTR). Primers (Fig. 1B) were designed at the 5' end of U3 (sense [P3], CTGCGGGGACGAC, position 7179 to 7193 [43]) and at the 3' end of U5 (antisense [P4], CTGCCGCGAC CAGCA, position 111 to 125 [43]). The buffer employed was 10 mM Tris (pH 8.9), 50 mM KCl, 2 mM MgCl₂, with 200 μM each dNTP, 100 ng of primers, and 1 U of *Taq* (Boehringer). PCR cycles employed were an initial step of 94°C for 2 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 2.5 min.

The products of three independent PCRs were pooled and then cloned in the TA cloning system (Invitrogen), according to the manufacturer's instructions. Three resultant clones were sequenced by the dideoxy chain termination method, employing a Li-Cor automated sequencer.

Heminested LTR-gag PCR. In the first round of amplification, 250 to 500 ng of tumor and kidney high-molecular-weight DNA was amplified by PCR, employing the Expand Long Template PCR system (Boehringer), which uses a mixture of *Taq* and *Pwo* DNA polymerases. The sense primer (P5, TGGGAGCTCTTTG GCAAAGCC, nucleotide position 7210 to 7224 [43]) was designed on the basis of nucleotide sequence differences between the endogenous LTR sequences (1) and the JSRV sequence (43). Primer P2 was employed as antisense primer (Fig. 1C). Reactions were carried out in a 50-μl volume containing 50 mM Tris-HCl (pH 9.2), 160 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 2% (vol/vol) dimethyl sulfoxide, 0.1% (vol/vol) Tween 20, 500 μM each dNTP, 300 nM each primer, template DNA, and 2.625 U of enzyme mix. Cycles were as follows: 1 cycle at 92°C for 2 min; 30 cycles at 92°C for 10 s, 61°C for 30 s, and 68°C for 90 s (plus a progressive increment of 20 s from cycle 11 onward); and a final extension of 68°C for 7 min.

One microliter of the product of the first round of amplification was used in the second round of PCR which was the same as the PCR-gag procedure described above. Half the product of the second-stage PCR was digested with *ScaI* as described above.

Products of the first and second rounds of heminested PCR were visualized by electrophoresis in 1 and 2% agarose gels, respectively.

Nucleotide sequence accession numbers. The nucleotide sequences of the PCR clones of *enJSRV gag* fragments and LTRs are deposited in GenBank under accession numbers Z49800, X92180, X92181, X92182 (*enJSRV gag1* to -4) and Z66531, Z66532, Z66533 (*enJSRV* LTRs 1 to 3).

RESULTS

Detection of *enJSRV gag* transcripts and differentiation between endogenous and exogenous transcripts. RT-PCR (*gag*), using the primer pair P1 and P2, was used to examine respiratory and other tissues from both SPA-affected and unaffected sheep for the presence of JSRV *gag* transcripts. The results are summarized in Table 1.

A product of the expected size (229 bp) was detected in all 65 sheep tissue samples tested. Primary cultures of fetal lamb and kidney cells produced the same visible amplicon, whereas dog lung tissues and an equine dermis cell line were consistently negative. To exclude DNA contamination in the total RNA preparations, the same samples were tested in parallel, omitting reverse transcriptase from the reaction mix; no signal was obtained from any of these samples.

The specificity of the amplicons was confirmed by Southern hybridization with an oligonucleotide probe from a region of JSRV *gag* internal to the PCR product (data not shown).

Previous studies have shown consistently that lung fluid and lung tumor tissues from SPA-affected sheep contain JSRV particles and antigens (18, 20, 27, 38); therefore, it may be assumed that the RNA detected in these materials is of exogenous origin, while the RNA detected from tissues of unaffected control animals is transcribed from endogenous sequences. RT-PCR using primers P1 and P2 therefore demonstrated that transcripts of endogenous origin are widely expressed in sheep tissues.

To identify possible differences between endogenous and exogenous RNA, the RT-PCR products obtained from kidneys of an unaffected control animal were cloned and sequenced. Seven clones were sequenced, and four different sequences (*enJSRV gag1* to -4) were obtained.

The four *enJSRV* sequences were compared with each other and with the published exogenous sequences of JSRV (43) (South African isolate) and pCA1 (19) (Peruvian isolate) by computer analysis using the GCG Pileup program (14) (Fig. 2). The South African and the Peruvian isolates were 90% similar in the stretch of sequence considered. The *enJSRV* clones were highly homologous to each other (95 to 99% similarity), 95 to 98% similar to the South African isolate, and 85 to 88% similar to the Peruvian isolate. Comparison of the restriction maps of the six sequences revealed a *ScaI* restriction site in JSRV and pCA1 that was absent in all the *enJSRV* clones. To confirm this observation, RT-PCR products from 16 lung fluid samples and 44 nontumor tissues were digested with *ScaI*. Successful digestion, indicated by the presence of two smaller fragments of 131 and 98 bp, was observed with all 16 lung fluid samples, 5 of 5 tumors, and faintly in 3 of 5 mediastinal lymph nodes from SPA-affected sheep. No *ScaI*-sensitive product was observed with any other tissue (Fig. 3).

These findings confirm the complete correlation between the *ScaI* site and the exogenous virus.

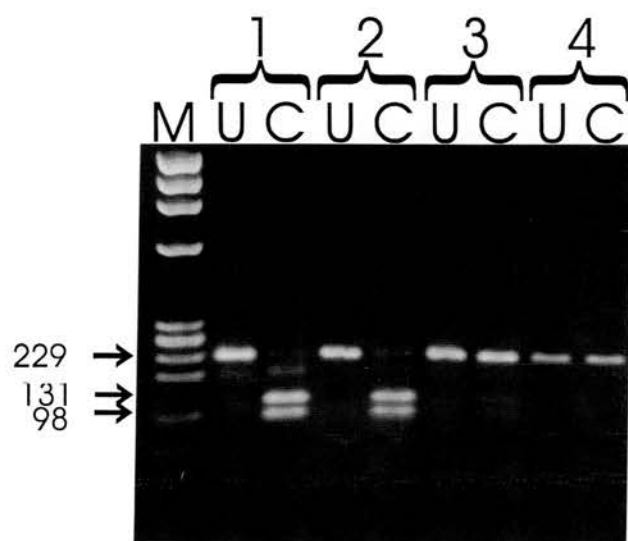


FIG. 3. Typical results of RT-PCR, followed by *ScaI* digestion, of total RNA from SPA-affected sheep tissues. Samples: 1, lung fluid; 2, lung tumor; 3, mediastinal lymph nodes; 4, kidney (samples 1 to 4 were collected from the same animal). Lanes: U, mock-digested PCR products; C, PCR product digested with *ScaI*; M, molecular weight marker IX (Boehringer).

Amplification and sequencing of endogenous LTRs. PCR products of approximately 450 bp were obtained from the amplification of DNA from cultured sheep primary skin fibroblast cultures by employing primers P3 and P4. Products were cloned as described in Materials and Methods, and three clones were completely sequenced (Fig. 4).

The *enJSRV* LTRs appeared to be longer (442 to 445 bp) than the published exogenous LTR (397 bp) which showed two large deletions of 30 and 16 bp at positions 198 and

261, respectively (nucleotide positions as in Fig. 4). The nucleotide sequences of the three *enJSRV* LTRs were 83 to 90% identical and were 69 to 76% similar to the JSRV LTR sequence (43).

Detection of exogenous JSRV in tumors of SPA-affected sheep. PCR products obtained by direct amplification of tumor genomic DNA with primers P1 and P2 followed by *ScaI* digestion did not show any cut product by electrophoresis in an agarose gel stained with ethidium bromide, and hybridization of Southern blots with an internal probe did not resolve this issue (data not presented). The failure to detect *ScaI*-sensitive sequences at the DNA level may be due to the high proportion of endogenous template compared with exogenous template, resulting in a small proportion of *ScaI*-sensitive product. Theoretically, there may be 20 endogenous copies compared with possibly only 1 exogenous copy per neoplastic cell. Moreover, neoplastic cells are approximately only 40 to 50% of the total number of cells present in the neoplastic tissue, therefore increasing the ratio of endogenous to exogenous sequences.

To preferentially amplify exogenous proviral DNA sequences (with the *ScaI* restriction site) we designed a hemi-nested PCR. The first round of amplification used a sense primer (P5) based on an area of sequence divergence between endogenous LTR clones and the exogenous LTR sequence (JSRV) and the antisense primer (P2) of the RT-PCR (*gag*). A product of about 2 kb was visible in an ethidium bromide agarose gel from three of four SPA tumor DNAs, while no signal was obtained in four of four kidney DNAs of the same animals or from lung and kidney tissues of three unaffected control animals. A second round of amplification, using primers of the RT-PCR (*gag*) followed by *ScaI* digestion, enabled the detection of *ScaI*-sensitive sequences in all four tumors examined, while only spurious bands were detected in nontumor samples of SPA-affected or nonaffected sheep (Fig. 5).

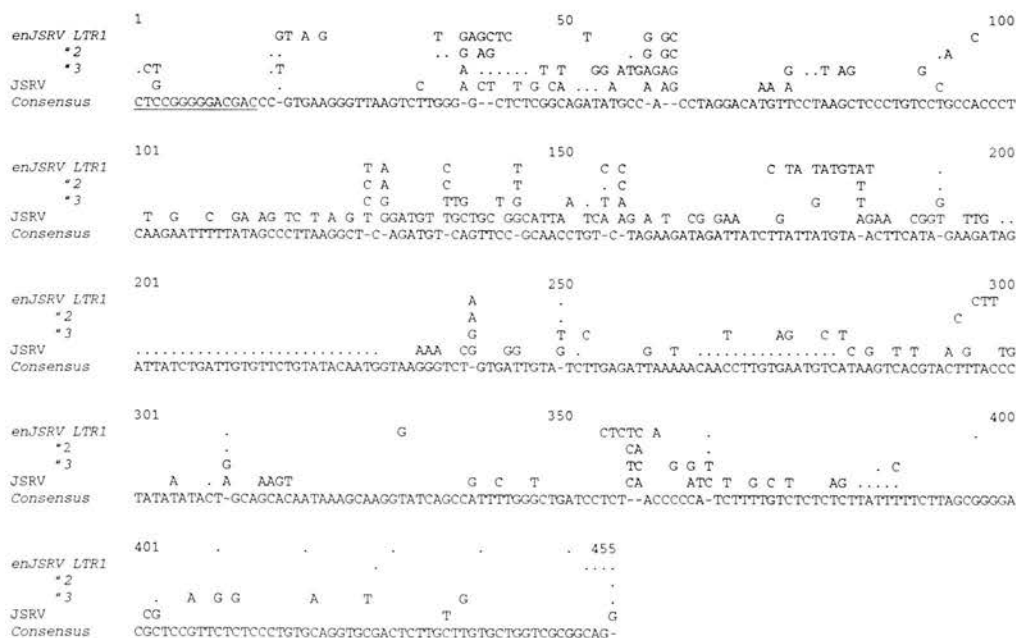


FIG. 4. Sequence alignment of *enJSRV* LTR clones with the JSRV LTR (43). Letters indicate differences with the consensus sequence. A period (.) indicates deletions. Primers used to obtain *enJSRV* clones are underlined.

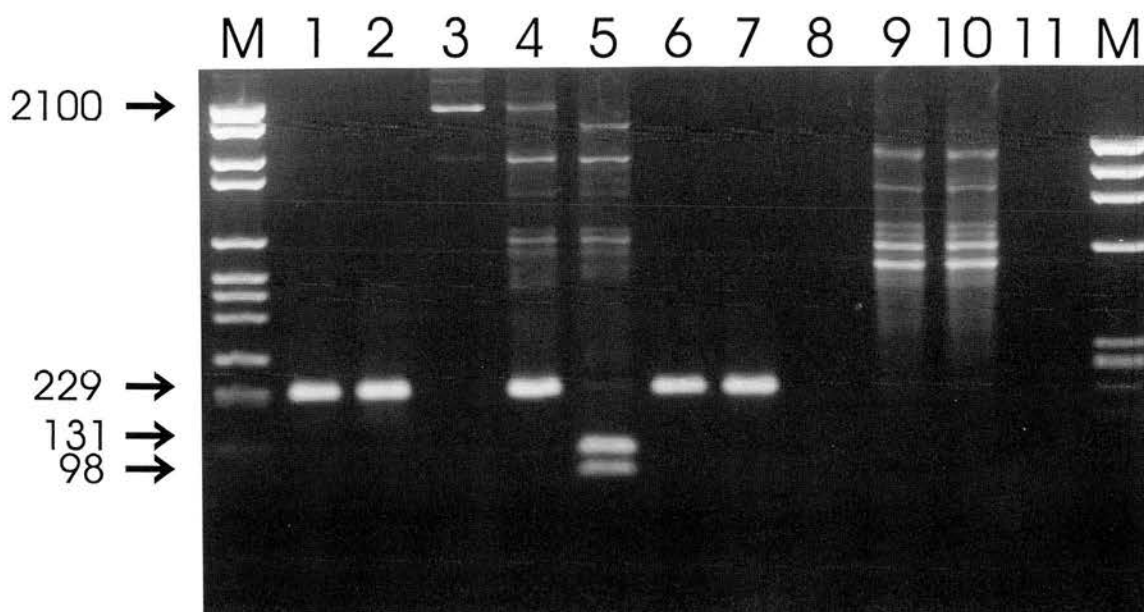


FIG. 5. Detection of exogenous JSRV sequences in tumor and kidney DNAs of the same SPA-affected sheep. Lanes 1 to 5 represent experiments performed with lung tumor genomic DNA. Lanes: 1, 500 ng of lung tumor genomic DNA amplified directly by PCR-*gag*, employing primers P1 and P2; 2, PCR product shown in lane 1 cut with *ScaI*; 3, first round of amplification of heminested LTR-*gag* PCR, employing primers P5 and P2; 4, second round of amplification, employing primers P1 and P2, of 1 μ l of the PCR product shown in lane 3; 5, PCR product of lane 4 cut with *ScaI*. Lanes 6 to 10 represent experiments performed with kidney genomic DNA. Lanes: 6, 500 ng of kidney genomic DNA amplified directly by PCR-*gag*, employing primers P1 and P2; 7, PCR product of lane 6 cut with *ScaI*; 8, first round of amplification of heminested LTR-*gag* PCR, employing primers P5 and P2; 9, second round of amplification, employing primers P1 and P2; 10, PCR product shown in lane 10 cut with *ScaI*. Lane 11 contains a negative control, lanes M contain Boehringer molecular weight markers VI (left) and IX (right).

DISCUSSION

This study has provided compelling lines of evidence which collectively demonstrate a specific association between the exogenous forms of JSRV and SPA.

It was shown clearly that the exogenous form of JSRV was distinct from endogenous JSRV transcripts as identified by the presence of a *ScaI* restriction site in *gag* (nucleotide position 1729 [43]). Furthermore, this site was conserved in strains of exogenous JSRV obtained from geographically dispersed countries, supporting data linking these viruses to each other and to SPA (18, 20, 27, 28, 33, 38).

Exogenous JSRV RNA was found only in tumors, lung fluid, and draining lymph nodes of SPA-affected sheep, which is in agreement with results of an earlier immunological study, which concluded that epithelial tumor cells are major sites of replication for JSRV (27). Lung fluid and tumor tissues from SPA-affected sheep are the only materials that have been used successfully to reproduce the tumor experimentally in sheep and goats (7, 24, 36, 37, 42). These findings, therefore, point to exogenous JSRV as a strong candidate for the etiological agent of SPA, although they do not rule out possibilities such as reactivation of an *enJSRV* as a downstream event of neoplasia. However, the demonstration of exogenous JSRV provirus only in tumor DNA provides irrefutable evidence that this virus is specifically associated with neoplasia. Endogenous JSRV transcripts were present in a wide variety of tissues from both SPA-affected and unaffected sheep, although downstream translation may be very low or absent, because CA protein was not detected in nontumor tissues (27).

Nevertheless, the presence of transcriptionally active *enJSRVs* raises a question about their potential role in SPA. In mice, chickens, and cats, endogenous retroviruses have been demonstrated to be involved in recombinational events leading to the generation of oncogenic retroviruses or viruses with a

broader host range (2, 12, 15, 25, 41). The possibility of recombinational events between the exogenous form of JSRV and its endogenous homologs cannot be ruled out. To clarify this point, further characterization of endogenous and exogenous JSRV sequences will be essential as limited sequence data are available (19, 43).

Comparison of LTR sequences between JSRV and three *enJSRV* clones derived from sheep skin fibroblasts revealed differences in putative enhancer sites. A putative NF-1 motif in exogenous JSRV LTR (nucleotides 7220 to 7223), which overlaps the exogenous-specific PCR primer P5, is altered and/or lost in the endogenous LTRs, and two tandemly repeated sequences (positions 7347 to 7365 and 7366 to 7384 in the JSRV sequence [43]) appear to be lost by means of an insertion of 30 nucleotides in the endogenous U3. Differences in the nucleotide sequences in these regions could potentially diminish the LTR function (8, 9, 11, 12, 17, 22, 23, 26, 31, 40) and thus reduce the pathogenicity of *enJSRVs* compared with the exogenous counterpart, as demonstrated in other retroviral systems.

An interesting finding of this study was the detection of exogenous JSRV RNA in three of five mediastinal lymph nodes of SPA-affected sheep. Previous immunological studies detected JSRV particles in only 1 of 12 regional lymph nodes of SPA-affected sheep (27). The detection of the exogenous JSRV in the majority of regional lymph nodes by RT-PCR may reflect the presence of metastases, although these were not detected by histological examination (data not shown), or may reflect the presence of the virus in the lymph drained by the regional lymph nodes at levels below the detection limit of the immunological techniques. Because of the lack of data on the immune response to JSRV, we cannot determine whether the presence of JSRV in the lymph nodes is related to an active immune response to the virus.

To date, JSRV has not been isolated *in vitro*, and therefore classical approaches to demonstrate the etiology of SPA have been impossible. However, the results reported in this communication show that exogenous JSRV is consistently and exclusively demonstrated in tumors and lung fluids, which have been used as inocula to induce SPA on numerous occasions (7, 24, 36, 42).

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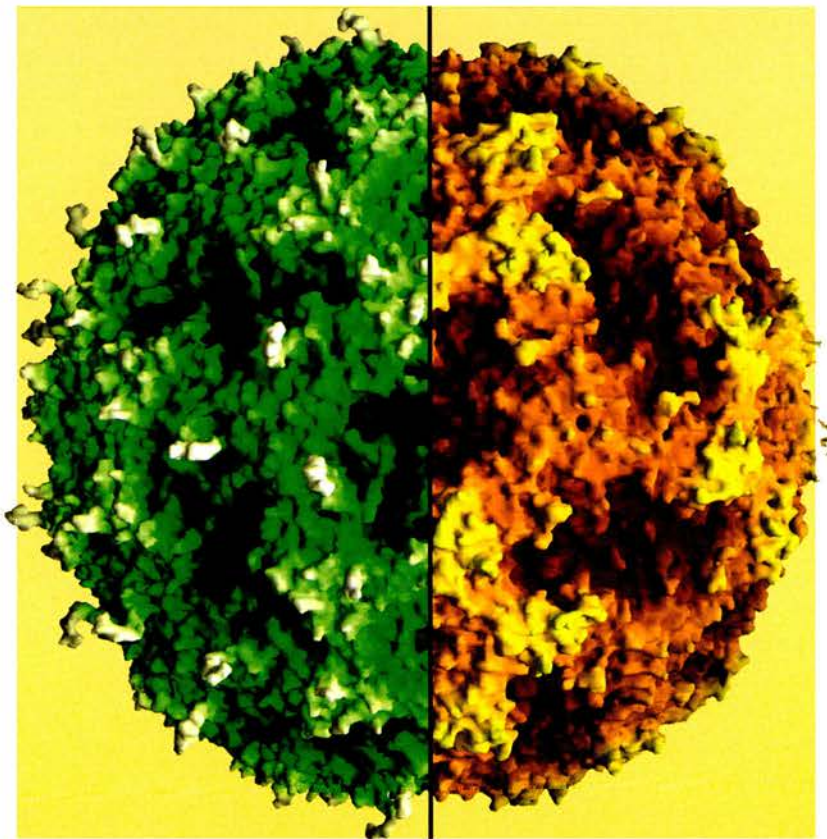
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Jaagsiekte retrovirus establishes a disseminated infection of the lymphoid tissues of sheep affected by pulmonary adenomatosis

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Jaagsiekte retrovirus (JSRV) is an exogenous type D-related retrovirus specifically associated with a contagious lung cancer of sheep (sheep pulmonary adenomatosis; SPA). Recently, epithelial tumour cells in the lungs of SPA-affected sheep were identified as major sites of JSRV replication by immunological techniques and RT-PCR amplification of part of JSRV *gag*. JSRV was not detected outside the lungs and their draining lymph nodes. However, low levels of JSRV expression in non-respiratory tissues could have been masked by co-amplification of endogenous JSRV-related sequences, which were differentiated from JSRV by the lack of a *Scal* restriction site in the PCR product. To further investigate the pathogenesis of SPA, an exogenous virus-specific hemi-nested PCR was developed utilizing primers in the U3 region of JSRV LTR, where major differences between endogenous and ex-

ogenous sequences exist. This technique was shown to be $\geq 10^5$ -fold more sensitive than the previous *gag* PCR/*Scal* digestion method. Using this new assay the tissue distribution of JSRV in sheep with natural and experimentally induced SPA was analysed. Proviral DNA and JSRV transcripts were found in all tumours and lung secretions of SPA-affected sheep ($n = 22$) and in several lymphoid tissues. The mediastinal lymph nodes draining the lungs were consistently demonstrated to be infected by JSRV (10/10). JSRV transcripts were also detected in spleen (7/9), thymus (2/4), bone marrow (4/8) and peripheral blood mononuclear cells (3/7). Proviral DNA was also detected in these tissues although in a much lower proportion of cases. JSRV was not detected in 27 samples from unaffected control animals ($n = 15$).

Introduction

Retroviruses isolated from mammalian species have been invaluable to our present knowledge of cancer initiation and progression. Animal models have been established, usually in murine and avian species, and have provided a better understanding of oncogenic events, particularly in cells of the lymphoreticular system (Weiss *et al.*, 1985; Fan, 1994). However, animal models of epithelial neoplasia associated with retroviruses have been restricted to mouse mammary tumour and the associated virus, mouse mammary tumour virus

(MMTV) (Gross, 1983). Such models of epithelial neoplasia are particularly relevant as tumours arising from epithelia are the most prevalent tumours in man (Silverberg *et al.*, 1990).

Sheep pulmonary adenomatosis (SPA; also known as jaagsiekte and ovine pulmonary carcinoma) represents a unique system because it is a naturally occurring epithelial neoplasm which is closely associated with a retrovirus (Verwoerd *et al.*, 1985; Sharp, 1987). Moreover, SPA shares certain characteristics with human bronchiolo-alveolar carcinoma (Perk & Hod, 1982; Gazdar & Linnoila, 1988) the aetiology of which is unknown and which is increasing dramatically in prevalence (Barsky *et al.*, 1994).

The virus associated with SPA, jaagsiekte sheep retrovirus (JSRV), cannot be cultivated *in vitro*. JSRV has morphological, biochemical and antigenic similarity to type D and type B retroviruses and has a genomic organization typical of a

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The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession number Z71304.

replication-competent retrovirus, containing no sequences commonly associated with transformation (Perk *et al.*, 1974; Sharp & Herring, 1983; Herring *et al.*, 1983; Rosadio *et al.*, 1988; York *et al.*, 1992).

Current data point to the involvement of JSRV in the aetiology of SPA, in particular, the experimental transmission of the disease only with inocula that contain JSRV (Martin *et al.*, 1976; Verwoerd *et al.*, 1980; Sharp *et al.*, 1983; DeMartini *et al.*, 1987) and the constant association of JSRV with transformed epithelial cells in the lungs of both naturally and experimentally SPA-affected sheep (Palmarini *et al.*, 1995). Recent molecular data have demonstrated that JSRV is an exogenous virus distinct from the transcriptionally active endogenous retroviral sequences present within the ovine genome [sheep endogenous retroviruses; SERVs (synonym: *enJSRV*)] (Palmarini *et al.*, 1996; Bai *et al.*, 1996; York *et al.*, 1992). A *ScaI* restriction site in *gag* has been defined as a molecular marker for the exogenous JSRV which has been consistently demonstrated in tumour and in draining lymph nodes of a proportion of SPA-affected sheep. JSRV was not detected outside the respiratory tissues of SPA-affected sheep nor in any tissue of control sheep (Palmarini *et al.*, 1996). Nevertheless, co-amplification of endogenous and exogenous sequences could have masked the detection of low copies of exogenous JSRV due to the relatively high background formed by SERVs.

To address this question, an exogenous JSRV specific hemi-nested PCR which amplified a segment of the U3 region of JSRV was designed. The anatomical distribution of JSRV in tissues collected from sheep with naturally and experimentally induced SPA was then investigated.

Methods

Animals. SPA-affected sheep were defined as animals with typical clinical signs, particularly the production of an abundant sero-mucoid fluid ('lung fluid') from the nostrils when the rear limbs were elevated above the head. Diagnosis was then confirmed by macroscopic and histological examination of the lungs. Seventeen naturally SPA-affected sheep and six experimentally infected lambs already utilized in a preceding study (Palmarini *et al.*, 1996) were used. Fifteen age- and breed-matched animals were selected as controls.

Preparation of samples. Samples of heparinized venous blood, lung fluid, lung, lung tumour, mediastinal lymph nodes, pre-scapular lymph nodes, mesenteric lymph nodes, spleen, femoral bone marrow, thymus, kidney and skin were collected during the post-mortem examination or (blood and lung fluid) immediately before the sheep were sacrificed. Sterile instruments were used to dissect each individual organ at post-mortem to avoid cross-contamination. Tissue samples were snap-frozen in liquid nitrogen and stored at -70°C . Lung fluid samples were clarified by centrifugation at $10\,000\text{ g}$ for 1 h at 4°C and then stored at -70°C . Plasma (5 ml) was clarified by centrifugation at $10\,000\text{ g}$ for 1 h at 4°C . The supernatant was then centrifuged at $100\,000\text{ g}$ for 1 h at 4°C . The pellet was resuspended in $500\text{ }\mu\text{l}$ of solution D (4 M-guanidinium thiocyanate, 25 mM-sodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M-2-mercaptoethanol) and stored at -20°C . Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by centrifugation over Lymphoprep at 800 g for 30 min; aliquots of 4×10^6 cells were stored at -70°C until RNA and DNA were extracted. Total RNA was extracted from $50\text{ }\mu\text{l}$ aliquots of lung fluid, 200–500 mg of lung tumour or from the plasma samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). Poly(A)⁺ RNA was obtained from 100 mg of all other tissues and from 4×10^6 PBMC using the Microfast Track kit (Invitrogen) according to the manufacturer's instructions. Genomic DNA was extracted from 10–25 mg of tissues and from 4×10^6 PBMC using the Qiagen Tissue Amp kit following the manufacturer's instructions.

Amplification and sequencing of exogenous LTR sequences. Sequences and nucleotide positions of the oligonucleotide primers relative to the JSRV genome (York *et al.*, 1992) are indicated in Table 1. The 5' LTR of a UK strain of JSRV was amplified from 500 ng of lung tumour genomic DNA isolated from a natural case of SPA using primers P-I and P-II. These formed the first primer pair of a hemi-nested LTR-*gag* PCR used to amplify proviral JSRV (Palmarini *et al.*, 1996). Briefly, the forward primer (P-I) is located almost at the 5' end of the U3 while the reverse primer anneals to *gag* (P-II) so that all but the first 33 bp of the JSRV 5' LTR are amplified. Previously, primer P-I was demonstrated to be specific for the exogenous form of JSRV (Palmarini *et al.*, 1996). The PCR products of three independent reactions were pooled and cloned in pGEM-T (Promega) as described by the manufacturer. Six selected clones (pGEM-T LTR-*gag*) were demonstrated to be of exogenous origin by the presence of a unique *ScaI* site in *gag* (data not shown). The region corresponding to the LTR of JSRV was then sequenced in both directions by the dideoxynucleotide chain termination method, employing a Li-Cor automated sequencer.

cDNA synthesis. cDNA synthesis was carried out using total RNA derived from ultraspeed pellets of plasma (5 ml) or $50\text{ }\mu\text{l}$ of clarified lung

Table 1. Oligonucleotide primers employed in the PCRs and hybridizations of this study

| Primers | Sequence (5' → 3') | Position* |
|---------|-------------------------------------|-----------|
| P-I | TGGGAGCTCTTTGGCAAAAGCC | 7210–7224 |
| P-II | ATACTGCAGCYCGATGGCCAG | 1806–1826 |
| P-III | CACCGGATTTTACACAATCACCGG | 7361–7385 |
| P-IV | GGCACTGCTTCACAGAAATATCAGGAAATCTGATT | 7316–7350 |
| P-V | TTTTTAAAAGCTCTTAAGGCTCGGATGTTTGCTT | 7280–7313 |
| P-VI | TGATATTTCTGTGAAGCAGTGCC | 7316–7338 |

* Nucleotide positions refer to the JSRV sequence published by York *et al.* (1992).

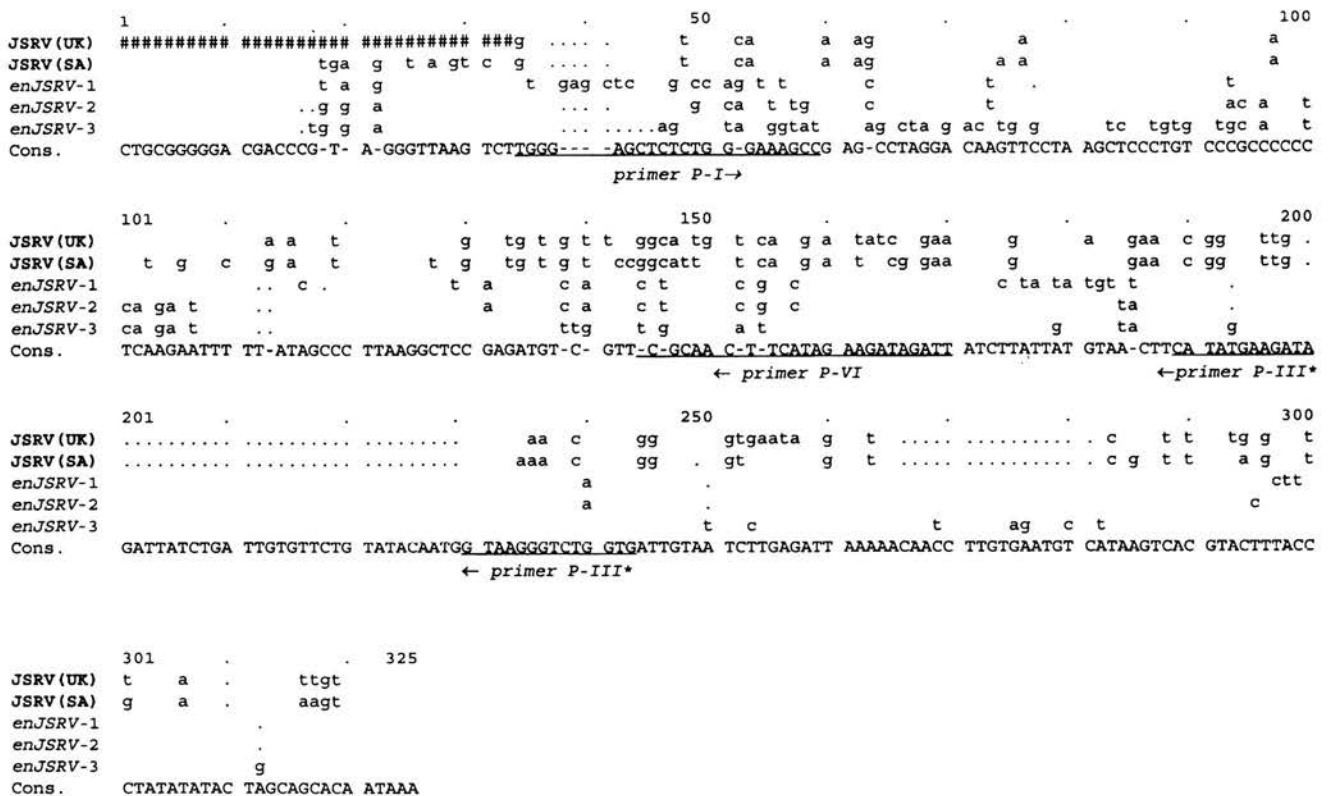


Fig. 1. Sequence alignment of the U3 of SERVs (Palmarini *et al.*, 1996), of the South African strain of JSRV [JSRV(SA)] (York *et al.*, 1992) and of a UK strain of JSRV [JSRV(UK)]. Letters indicate differences with the consensus sequence. A period (.) indicates deletions. Lack of sequence data in the JSRV(UK) sequence is indicated with (#). Positions of the primers employed in the JSRV U3-PCR and hn-U3 PCR are underlined. P-III (asterisk) appears discontinuous only in the figure due to the 30 bp deletions of JSRV sequences with respect to SERV (*enJSRV*).

fluid as template. For analysis of other samples, 2 µg of total RNA from tumour samples or 1 µg poly(A)⁺ RNA from other tissues and PBMC were used as template. RNA was diluted in 13.5 µl of diethyl pyrocarbonate-treated distilled water and denatured at 65 °C for 10 min and cooled on ice. One microlitre (40 U) of rRNasin (Promega), 2 µl of 25 mM each deoxynucleoside triphosphate (dNTP), 2.5 µl of 100 mM-DTT, 2.5 µl random hexamers (*A*₂₆₀ 62.5 U/ml; Oswel), 2.5 µl of 10 × reaction buffer (500 mM-Tris-HCl pH 8.3, 75 mM-KCl, 3 mM-MgCl₂) and 1 µl of MoMuLV reverse transcriptase (50 U/µl; Stratagene) were added. Reverse transcriptase (RT) was omitted in control samples to check for DNA contamination of the RNA preparations. The reaction mixture was then incubated at 37 °C for 1 h, and the RT inactivated by incubation at 94 °C for 3 min.

■ **Exogenous-specific JSRV U3 and U3 hemi-nested (hn) PCR.** Primer P-I was paired with an antisense primer (P-III) in the U3

Primer 1 was paired with an antisense primer (Primer 2) in the U3 region at nucleotide positions 7385–7361 (York *et al.*, 1992) where there are major sequence differences between JSRV and SERVs. In this region the endogenous sequences carry a 30 bp insertion compared to the exogenous sequences (Fig. 1). The JSRV U3-PCR reaction was optimized using DNA from a LTR-*gag* pGEM-T plasmid as template. The buffer used was 10 mM-Tris-HCl, 50 mM-KCl, 2.5 mM-MgCl₂ (pH 9.2) with 200 µM each dNTP, 6.25 pmol of each primer and 1.25 U of *Taq* polymerase (Boehringer) in a 50 µl reaction. Five hundred nanograms of genomic DNA or 3 µl of cDNA were used as template in the reaction. Each sample was subjected to 94 °C for 1 min followed by 35 cycles of

94 °C for 30 s, 59 °C for 1 min and 72 °C for 1 min with a final extension of 72 °C for 3 min, using a Perkin Elmer GeneAmp 2400 thermal cycler.

Total RNA extracted from 22 lung tumour and lung fluid samples from 16 different naturally acquired SPA cases and 4 experimentally induced cases was tested to verify the ability of JSRV-U3 PCR to detect different isolates of JSRV. In addition, genomic DNA extracted from lung tumour and matched kidney samples of four sheep with SPA was tested to verify that JSRV U3-PCR does not amplify SERV sequences.

To verify the specificity of the PCR products, 25 μ l of the PCR product was resolved by electrophoresis in 1.5% agarose gels and transferred to nylon membranes overnight by established procedures (Sambrook *et al.*, 1989). Membranes were then fixed, pre-hybridized and probed overnight at 42 °C with 10 pmol each of two digoxigenin (DIG) 5'-labelled oligonucleotides (P-IV and P-V; Oswel) in DIG Easy Hyb (Boehringer) hybridization buffer. The hybridized probe was then detected using the DIG Nucleic Acid Detection Kit (Boehringer) as recommended by the manufacturers.

To improve the sensitivity of the JSRV-U3 PCR, a primer internal to the PCR product, primer P-VI, was synthesized and paired with primer P-I in a second round hemi-nested PCR (JSRV U3-hn PCR). One microlitre of the product of the first round of amplification was added to 49 μ l of 1 \times PCR buffer [1.25 U of *Taq* polymerase (Boehringer), 2.5 mM-MgCl₂, 50 mM-KCl, 10 mM-Tris-HCl, 200 μ M each dNTP and 6.25 pmol of each primer]. PCR cycles employed were 94 °C for 1 min and 35 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min, with a final

extension of 72 °C for 3 min. Amplified products were detected by electrophoresis of 25 µl aliquots through a 2% agarose gel containing 0.5 µg/ml of ethidium bromide.

The sensitivities of the JSRV U3-PCR and U3-hn PCR, against a background of endogenous-related sequences, were compared with each other and with the previously described JSRV *gag* PCR followed by *ScaI* digestion (Palmarini *et al.*, 1995). 'Reconstruction' experiments were performed using serial dilutions (10^5 to 10^{-1} copies) of clone pGemT-LTR-*gag* (which contains the U3 through *gag* of JSRV provirus) with and without the addition of 500 ng of normal sheep kidney DNA as a source of SERV sequences. In addition, 100 copies of pGEM-T LTR-*gag* plasmid, plus serial dilutions (10^2 – 10^7 copies) of SERV-LTR or SERV-*gag* plasmids were tested.

Contamination of PCR was minimized by the use of appropriate controls in each step of the work. Isolation of nucleic acids, preparations of PCR reactions and analysis of PCR products were conducted in separate rooms.

■ Template control PCRs. Each cDNA sample which was negative after the U3-hn PCR was tested for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts to verify the integrity of the RNA template. Primers were designed on a consensus sequence between the rat and human GAPDH (C. McInnes, Moredun Research Institute, Edinburgh, personal communication). The sense primer (5' TCACCACCATGGAGAAGGCT 3') was based on exon 4 of the human gene and the reverse primer was based on exon 7 (5' TTCATTGTCATACCAGGAAA 3') (Tokunaga *et al.*, 1987). The buffer employed was 10 mM-Tris-HCl, 50 mM-KCl, 2.5 mM-MgCl₂ (pH 8.9) with 200 µM each dNTP, 6.25 pmol each primer and 1.25 U *Taq* polymerase (Boehringer). Cycles employed were 94 °C for 1 min and then 35 cycles of 94 °C for 45 s, 52 °C for 1 min and 72 °C for 1 min 30 s with a final extension of 72 °C for 5 min.

Parallel samples in which the addition of RT was omitted in the cDNA synthesis step were also tested to rule out DNA contamination. Amplified products were detected by electrophoresis of 25 µl aliquots through 1% agarose gels in 1 × TBE buffer in the presence of 0.5 µg/ml ethidium bromide. A DIG-labelled internal probe (5' DIG-CTCATGACCACAG-TCCATGCCATCAGCC) was used to confirm the specificity of the amplified product. Southern blotting and detection of hybridized probe were essentially as described above for U3-PCR. DNA samples which were negative after hn-U3 PCR were checked by amplification of a portion of SERV *gag* as previously described (Palmarini *et al.*, 1996).

Results

Sequencing of JSRV LTR and designing of JSRV exogenous-specific primers

To better design an exogenous virus-specific PCR the LTR of a UK strain of JSRV was sequenced and compared with the sequence of a South African strain of JSRV and the LTR of SERVs (York *et al.*, 1992; Palmarini *et al.*, 1996). The LTR of the UK strain of JSRV [JSRV(UK)] was 89% homologous to the LTR of the South African strain [JSRV(SA)] (York *et al.*, 1992) and 76–79% similar to SERV loci (Palmarini *et al.*, 1995). Major sequence divergence between exogenous and endogenous viruses was confirmed in the U3 region. In particular, a 30 bp deletion in the U3 of JSRV(SA) compared to the endogenous loci was also confirmed in JSRV(UK). Primer P-III was designed across the deletion such that at the optimal annealing temperature this primer should anneal efficiently only with the

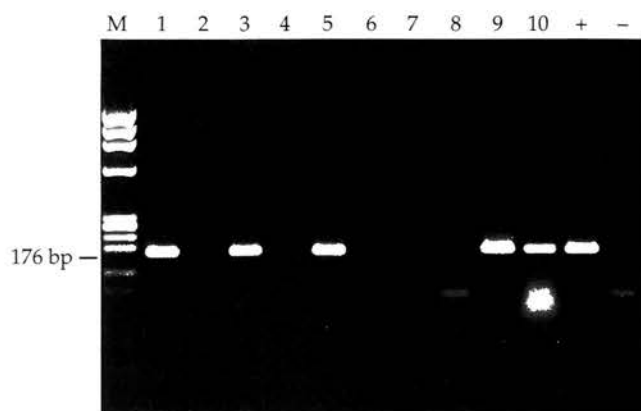


Fig. 2. Specific amplification of JSRV by U3-PCR. Lanes 1, 3 and 5 are the product of amplification of 500 ng of genomic DNA from three cases of SPA; lanes 2, 4 and 6 are the matched kidney samples. Lanes 7 and 8 are cDNA of lung of healthy control sheep; lane 9 is lung tumour cDNA and lane 10 is lung fluid cDNA. Symbols '+' and '-' indicate pGEM-T LTR-*gag* and water, respectively, used as positive and negative controls. Lane M contains molecular mass marker IX (Boehringer).

exogenous sequences. The alignment of the U3 of JSRV and SERV sequences and the relative positions of the primers employed in this study are indicated in Fig. 1. The JSRV LTR sequence of the UK strain has been deposited in GenBank under accession number Z71304.

Specificity and sensitivity of JSRV U3 and hemi-nested U3 PCRs

Twenty-two cDNA samples of lung fluid and lung tumour were examined by PCR using primers P-I and P-III. A product of the expected size of 176 bp was obtained with all samples. Hybridization with the DIG-labelled internal oligonucleotides P-IV and P-V confirmed that the products originated from JSRV-LTR (data not shown). To demonstrate the PCR product was derived only from JSRV and not from SERV, matched genomic DNA samples of lung tumour and kidney from four SPA-affected animals were examined. An amplified product was obtained from tumour DNA and not from the corresponding kidney sample, proving that the JSRV-U3 PCR is exogenous virus specific (Fig. 2).

The sensitivity of the U3-PCR was determined by 'reconstruction' experiments, testing mixtures of exogenous JSRV and SERV templates described in Methods. JSRV U3 PCR successfully amplified 10^2 – 10^3 JSRV template copies in a background of 500 ng of normal sheep genomic DNA and 10^2 JSRV template copies in a background of 10^7 copies of SERV-LTR.

The sensitivity of the U3-PCR was increased by using primer P-VI and primer P-I in a second round PCR. A product of 133 bp was detected from the re-amplification of all the lung fluid and lung tumour PCR products but not from the kidney samples. The U3-hn PCR detected an estimated single molecule of template in a background of 500 ng of normal sheep

Table 2. Results of the JSRV U3-hn PCR on tissues of sheep affected by SPA

Results are given as number of samples positive of the number tested. In all cases RNA refers to poly(A)⁺ selected RNA and DNA to genomic DNA.

| Tissues | Natural SPA | | Experimental SPA | | Total | |
|-------------------------|-------------|-----|------------------|-----|-------|-------|
| | RNA | DNA | RNA | DNA | RNA | DNA |
| Lung tumour/lung fluid | 18/18 | 6/6 | 4/4 | 4/4 | 22/22 | 10/10 |
| Mediastinal lymph nodes | 6/6 | 6/6 | 4/4 | 3/3 | 10/10 | 9/9 |
| Other lymph nodes | 2/9 | 0/9 | NA | NA | 2/9 | 0/9 |
| Spleen | 4/5 | 0/5 | 3/4 | 2/3 | 7/9 | 2/8 |
| Bone marrow | 2/5 | 2/4 | 2/3 | 1/3 | 4/8 | 3/7 |
| Thymus | NA | NA | 2/4 | 0/4 | 2/4 | 0/4 |
| Kidney | 1/6 | 1/6 | 0/3 | 0/3 | 1/9 | 1/9 |
| Skin | 0/4 | 0/4 | NA | NA | 0/4 | 0/4 |
| Plasma | 0/2 | / | 0/6 | / | 0/8 | / |
| PBMC | 3/7 | 0/6 | NA | NA | 3/7 | 0/6 |

NA, Not available

genomic DNA. In contrast, *gag* PCR followed by *ScaI* digestion was at least 10⁵-fold less sensitive than the U3-hn PCR because 10⁵ copies of pGem-T LTR-*gag* (exogenous clone) were not detected against a background of 500 ng of normal sheep kidney DNA. Furthermore, exogenous sequences were detected only when the ratio of exogenous/endogenous sequences was 1:10 but not \geq 1:100.

The U3-hn PCR amplified only exogenous sequences and was specific for different strains of JSRV.

Detection of proviral DNA and JSRV transcripts in non-tumour tissues of SPA-affected sheep

Following the successful development and validation of the U3-hn PCR, the anatomical distribution of JSRV in SPA-affected sheep was analysed. Table 2 summarizes the results obtained.

JSRV RNA and proviral DNA was found consistently in the mediastinal lymph nodes of SPA-affected sheep (10/10). In addition, JSRV RNA was found in 7/9 spleens, 4/8 bone marrows and 2/4 thymuses (Fig. 3). Proviral DNA was found more rarely in these tissues. PCR products were detected in 2/8 spleens and 3/7 bone marrows. Among the non-regional lymph nodes analysed all were negative for proviral DNA, but two mesenteric lymph nodes of the five analysed were positive at the RNA level. Pre-scapular lymph nodes (*n* = 4) were always negative. PBMC were found to be positive for JSRV RNA in 3/7 samples but proviral DNA was not detected in any. Plasma samples were always negative.

Apart from lymphoid tissues, JSRV was detected at both the DNA and RNA level in only one kidney of nine tested and in none of four skin samples. No differences were observed in the anatomical JSRV distribution between the naturally acquired and the experimentally induced SPA cases.

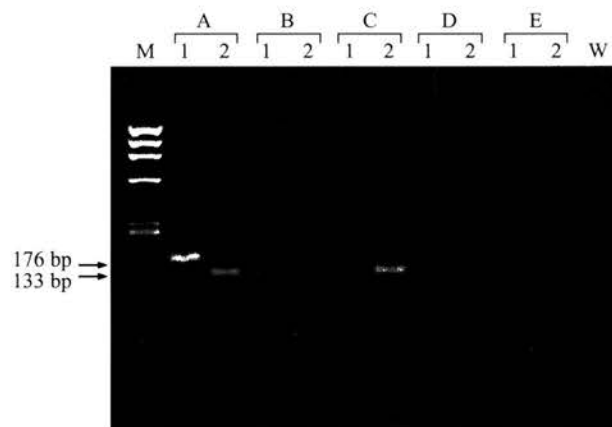


Fig. 3. Example of the results of JSRV U3-hn PCR in a SPA-affected animal. Lanes 1 contain the first step U3 PCR product and lanes 2 contain the second round of amplification (U3-hn PCR). Lanes A, lung tumour cDNA; lanes B, kidney cDNA; lanes C, spleen cDNA (note the faint product obtained in the first step); lanes D skin cDNA; lanes E, lung cDNA from an unaffected control animal. Lane M contains molecular mass marker IX (Boehringer). W is the negative control (water) of the hn-U3 PCR.

JSRV was not detected in 27 samples examined from 15 unaffected control animals. These samples included poly(A)⁺ RNA prepared from lung (*n* = 5), mediastinal lymph nodes (*n* = 7), thymus (*n* = 1) and PBMC (*n* = 4). In addition, samples from lung (*n* = 3), mediastinal lymph nodes (*n* = 1), spleen (*n* = 2), thymus (*n* = 1), bone marrow (*n* = 1) and kidney (*n* = 2) were tested for proviral DNA.

All the samples that were negative in the U3-hn PCR were positive for the amplification of GAPDH cDNA or SERV *gag* DNA, demonstrating that the templates were not degraded, and PCR inhibitors were not present after the nucleic acid extraction.

Discussion

Previous studies, using immunological and molecular techniques, demonstrated that JSRV is an exogenous virus, which does not arise from reactivation of an endogenous locus (Palmarini *et al.*, 1995, 1996). These studies identified the lungs, particularly the epithelial tumour cells, as a major site of JSRV replication, although viral RNA was demonstrated in the draining mediastinal lymph nodes of a proportion of SPA-affected sheep. These observations have been extended in the present report as a result of developing a highly sensitive assay for exogenous JSRV and show that JSRV RNA and proviral DNA is present not only in the draining lymph nodes but also in several anatomically dispersed lymphoid tissues and PBMCs. This distribution of JSRV in infected animals is, therefore, similar to that reported for other type B and type D retroviruses, in which both lymphoid and non-lymphoid tissues are infected (Bentvelzen & Brinkhof, 1977; Kozma *et al.*, 1980; Bryant *et al.*, 1986; Lackner *et al.*, 1988).

Although quantitative assays were not employed in the present studies, the estimated sensitivity of the different PCR assays confirmed that JSRV was expressed at much higher levels in SPA tumours than in the lymphoid tissues, particularly those at the non-local sites. Thus, there are clear parallels with the high levels of MMTV transcription associated with the development of breast carcinoma in mice (Varmus *et al.*, 1973; Henrard & Ross, 1988; Bramblett *et al.*, 1995). The transcriptional activity of MMTV appears to be determined by the interaction of a number of factors involving regulatory elements within the LTR, including at least one region implicated in elevated transcription in the mammary epithelium (Lefebvre *et al.*, 1991; Mink *et al.*, 1992; Mok *et al.*, 1992), and the absence of tissue-specific factors such as negative regulatory element binding protein (NBP) in mammary tissue (Bramblett *et al.*, 1995). Similar putative regulatory sequences, such as NF-1- and C/EBP-binding sites, have been identified in the JSRV LTR (York *et al.*, 1992) as well as many point mutations and large deletions compared to endogenous loci (Palmarini *et al.*, 1996; Bai *et al.*, 1996). Other retroviruses with LTR deletions have been reported to have a transcriptional advantage (Hsu *et al.*, 1988) and the biological significance of these structures in JSRV awaits the development of permissive culture systems.

The interaction between type B and type D retroviruses and lymphoid cells has been demonstrated to be an important feature in the pathogenesis of these viruses. B lymphocytes have a central role in the pathogenesis of MMTV and infection of the mammary gland requires a functional immune system. MMTV initially appears to infect B lymphocytes and is then delivered to the mammary gland through T cell-B cell interactions (Tsubura *et al.*, 1988; Golovkina *et al.*, 1992; Held *et al.*, 1994; Matsuzawa *et al.*, 1995). The simian type D retrovirus, SRV1, which induces simian acquired immunodeficiency syndrome in macaques (Gardner *et al.*, 1988), has a

broad *in vivo* lymphoid tropism involving lymphocytes and mononuclear cells. Infection leads to both T and B lymphocyte depletion although monocyte and macrophage function appears to be unaffected (LeGrand *et al.*, 1985; Maul *et al.*, 1988).

The significance of infection of lymphoid tissues by JSRV in the overall pathogenesis of SPA is unknown at present. As only sheep with clinical SPA were examined it is not known whether lymphoid infection precedes, or is a sequel to, JSRV replication in the pulmonary epithelium. Further studies will be required to define which cell types are involved and at which stages during the protracted time between infection and the appearance of clinical illness. Similarly, it is not clear whether sheep infected with JSRV develop immunodeficiency, although this possibility is supported by anecdotal reports of the increased susceptibility to secondary bacterial pneumonias (Sharp & Martin, 1983; Verwoerd, 1990) and lymphopenia in SPA-affected sheep (Rosadio & Sharp, 1992). The apparent absence of circulating antibodies to JSRV in sheep with SPA has been reported (Sharp & Herring, 1983; Verwoerd, 1990) although a recent study, using a recombinant Mason-Pfizer monkey virus major capsid protein, has produced conflicting results (Kwang *et al.*, 1995).

One of the major obstacles to progress in research on JSRV has been the lack of an *in vitro* culture system. The present study has clearly demonstrated permissive infection of lymphoid cells by JSRV, cell types which have been used successfully for the propagation of many retroviruses and which, therefore, could provide a permissive substrate for JSRV replication.

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PCR-Based Detection and Partial Characterization of a Retrovirus Associated with Contagious Intranasal Tumors of Sheep and Goats

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A type D-related retrovirus has been demonstrated in enzootic nasal tumors (ENTs) of sheep and goats. This retrovirus, ENT virus (ENTV), has antigenic cross-reactivity with the jaagsiekte sheep retrovirus (JSRV), which is associated with a contagious lung tumor of sheep (sheep pulmonary adenomatosis). Here, we present the first report of nucleic acid sequence from ENTV which confirms, at the nucleic acid level, that this retrovirus is related to JSRV yet apparently distinct from it. Reverse transcription-PCR followed by restriction enzyme digestion specifically identified ENTV. By this technique, ENTV was demonstrated exclusively in tumor tissues and exudates of animals with ENT. Thus, there is a unique and consistent association between ENT and the retrovirus, just as there is between JSRV and sheep pulmonary adenomatosis. This gives further weight to the hypothesis that these retroviruses are the etiologic agents of the tumors.

Enzootic intranasal tumors (ENTs) of sheep and goats are contagious adenocarcinomas which can also be transmitted experimentally (3, 6). ENTs have similarities with another contagious tumor of secretory epithelial cells, known as sheep pulmonary adenomatosis (SPA, or jaagsiekte ovine pulmonary carcinoma). Both tumors are associated with retroviruses which cannot be propagated *in vitro*. Although no studies have previously been reported to determine whether ENT and SPA are different manifestations of disease caused by the same agent, studies of ENT cases have not found any tumors in the lungs (7, 19), nor have nasal tumors in SPA-affected animals been reported.

In the case of SPA, an association with a type B/D retrovirus, known as jaagsiekte sheep retrovirus (JSRV), is well established (15, 17), and the complete nucleotide sequence of the retrovirus has been determined (22). Recent reports have demonstrated that JSRV is related to sheep endogenous retroviruses (SERVs), of which there are 10 to 15 copies in the sheep genome (1, 12, 15). However, reverse transcription-PCR (RT-PCR)-based techniques, which differentiate JSRV from SERVs, showed that JSRV is a distinct exogenous virus that is associated exclusively with SPA tumors (15).

Although studies are at a less advanced stage than for SPA and JSRV, current evidence points to the association of a type D-like retrovirus, ENT virus (ENTV), with ENT. The presence of retrovirus-like particles in ENT of sheep (14, 21) and goats (7, 19) has been demonstrated by electron microscopy. The detection of RT activity in fractions from isopycnic gradients has shown that the retrovirus found in nasal fluid of ENT-affected goats has the same buoyant density as JSRV (9). In addition, antigenic cross-reactivity with Mason-Pfizer monkey virus p27 (8) and recombinant JSRV capsid protein (unpub-

lished observation) indicates that ENTV has a type D retroviral capsid.

The aim of our studies was to develop a PCR-based detection technique for ENTV, to confirm its association with ENT, and to determine the relationship between ENTV and JSRV.

MATERIALS AND METHODS

Source of material. Nasal fluids from four sheep and two goats with naturally occurring ENT were collected, clarified, and concentrated as described previously (8). The resultant pellets were resuspended as a 100-fold concentrate in TNE buffer (0.01 M Tris-Cl [pH 7.5], 0.1 M NaCl, 0.001 M EDTA). Samples of ENT and of kidney tissues from the same animals were collected at necropsy and snap frozen in liquid nitrogen. The ENTs were all low-grade adenocarcinomas, which were locally invasive but not metastatic, and were from 3- to 4-year-old animals. Lung fluids from SPA-affected animals were collected as described previously (18). All samples were stored at -70°C. The ENT-affected sheep were verified to be negative for SPA by macroscopic and microscopic pathology at necropsy. The ENT-affected and SPA-affected cases used in this study were from the same region of Spain. The negative-control animals were from the United Kingdom, where ENT has never been reported.

Density gradient fractionation. Pellets from nasal fluids of four sheep and two goats were analyzed by isopycnic centrifugation on 20 to 55% (wt/wt) sucrose gradients (13). Fractions (0.5 ml) were collected, and those with densities between 1.17 and 1.19 g/ml (i.e., those fractions shown to contain peak RT activity [9]) were pooled, diluted in TNE buffer, and centrifuged at 100,000 × g for 1 h at 4°C. The pellet was resuspended in 0.5 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl [wt/vol], 0.1 M 2-mercaptoethanol) for RNA preparation.

RNA preparation and RT. Total RNA was extracted from pooled sucrose gradient fractions, from 100 µl of concentrated nasal fluid, from 100 µl of SPA lung fluid, or from 0.2 to 0.5 g of tissue by the acid guanidinium thiocyanate-phenol-chloroform method (2). cDNA was synthesized by random-primed RT as described previously (15).

gag PCR. The primers and conditions used for PCR of cDNA or of genomic DNA were exactly as described previously (15): P1 (sense), 5'-GCTGCTTTTACACCTTATCGAAA-3'; and P2 (antisense), 5'-ATACTGCAGCYCGATGGC CAG-3'. PCR products were detected by electrophoresis through a 2% agarose gel in 1× Tris-borate-EDTA buffer in the presence of 0.5 µg of ethidium bromide per ml.

Cloning of ENTV gag fragments. cDNA from nasal fluid from each of three ovine ENT cases was amplified by using primers P1 and P2. The products of four independent PCRs for each animal were pooled, gel purified, and cloned into pGEMT (Promega) according to the manufacturer's protocol. Several clones selected from each ENT case were sequenced on both strands by using the T7 sequencing kit (Pharmacia), with universal and reverse sequencing primers (Pharmacia), as recommended by the manufacturer. The sequences obtained

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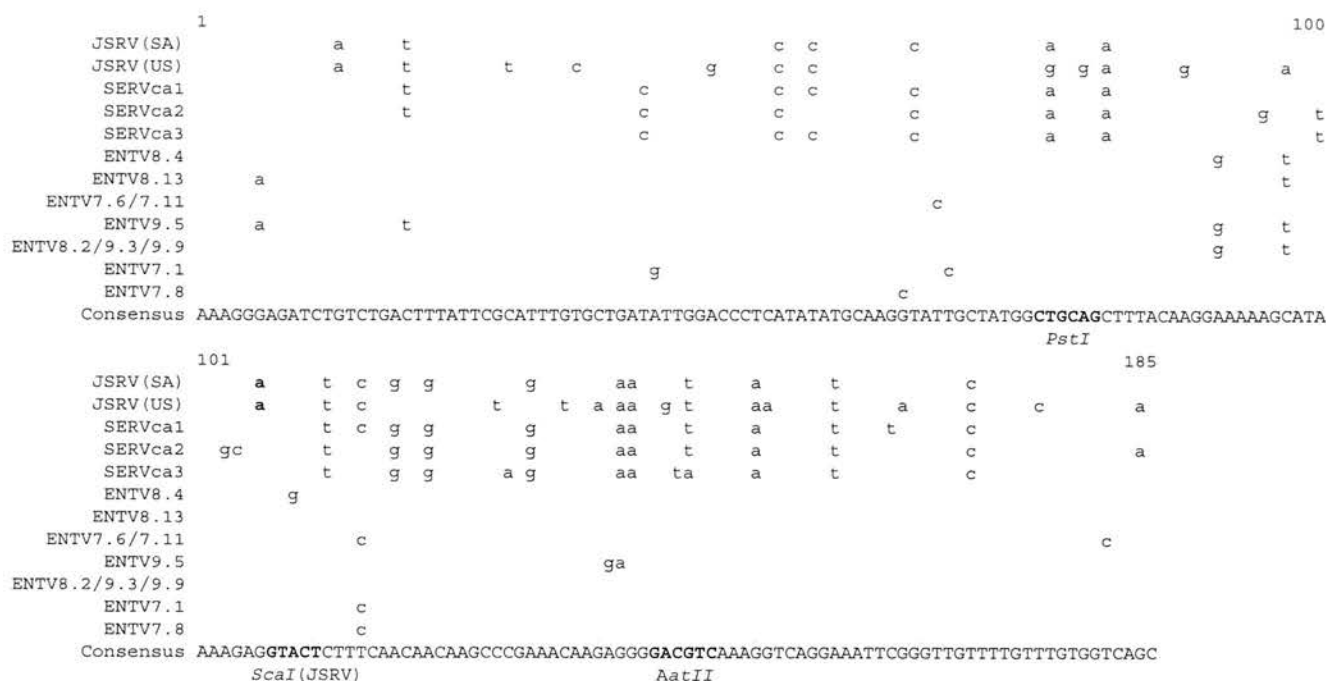


FIG. 1. Alignment of the sequences of RT-PCR *gag* products from ovine ENT with SERV sequences (15) and JSRV sequences from South Africa [JSRV(SA)] (22) and the United States [JSRV(US)] (12). Only the sequence between the PCR primers is shown. Base differences in comparison with the ENT consensus (letters) and restriction sites specific to ENT or JSRV (boldface) are shown.

were aligned by using the Genetics Computer Group "pileup" program (11). The phylogenetic tree was constructed by using the DNAML program from version 4.0 of the PHYLIP package (10).

Restriction endonuclease digestion of RT-PCR products. A 3- μ l volume of 10 \times restriction buffer (Boehringer) and 1 μ l (10 U) of restriction endonuclease (Boehringer) were added to 26 μ l of PCR product, and the mixture was incubated at 37°C overnight. Digestion products were detected by electrophoresis through a 2% agarose gel in 1 \times Tris-borate-EDTA buffer in the presence of 0.5 μ g of ethidium bromide per ml.

DNA extraction. High molecular weight genomic DNA was extracted from nasal tumor and kidney samples of the same naturally affected animals or from lung and kidney samples of unaffected controls by cell lysis followed by precipitation of DNA in tissue culture plates, as described by Wu et al. (20).

Nucleotide sequence accession numbers. The nucleotide sequence of one of the ENT *gag* fragment PCR clones, pENTV7.6, is deposited in GenBank under accession no. Z71758.

RESULTS

Development of a technique for specific detection of ENT.

Primers P1 and P2, which amplify a 229-bp region internal to the *gag* gene of JSRV and of SERVs (15), amplified products of the same size from cDNAs derived from clarified nasal fluids of four sheep and two goats affected by ENT and from density gradient-purified virus from the same sources. The *gag* RT-PCR product from JSRV isolates cut with *ScaI*, while that from SERVs or ENT did not (data not shown). To look for an alternative restriction site by which to distinguish ENT from SERVs, *gag* RT-PCR products of three ovine ENT cases, from different farms, were cloned and sequenced. Alignment of the sequences (Fig. 1) with the published sequences of JSRV and SERVs showed a number of bases at which there appear to be conserved differences between ENT and JSRV or SERV. Of particular interest were the presence of a *PstI* site (positions 75 to 80) and an *AatII* site (positions 141 to 146) unique to the ENT *gag* sequences and the lack of the *ScaI* site (positions 106 to 111) which has been shown to be present in all JSRV isolates tested to date (15).

Association of ENT with ENT. To confirm the association of the virus with ENT and the conservation of the *AatII* and *PstI* restriction sites, *gag* RT-PCR was done with 26 different samples (typical results are shown in Fig. 2). The product from nontumor (kidney) tissues of four of the ENT-affected animals or of four unaffected controls did not cut, as was expected for endogenously derived amplimers. The product from JSRV, in lung fluid or tumor samples from SPA-affected sheep, also did not cut with *AatII* or *PstI*. However, the RT-PCR *gag* products

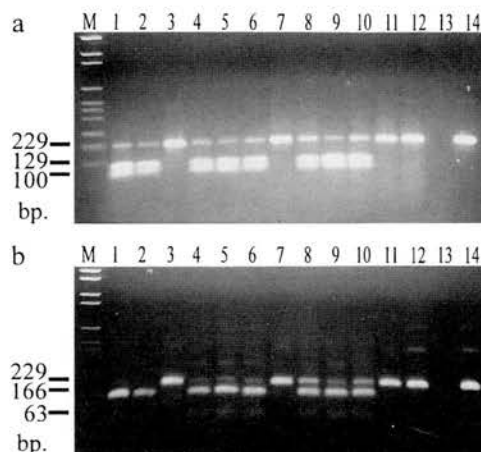


FIG. 2. Detection of ENT transcripts. *gag* RT-PCR products after incubation with *PstI* (a) or *AatII* (b) are shown. Lanes 1, 2, 4, and 5, nasal fluids from ENT-affected sheep; lanes 6, 8, 9, and 10, tumor tissues from ENT-affected animals; lanes 5 to 7, nasal fluid, tumor, and kidney samples, respectively, from the same ENT-affected sheep; lanes 3, 11, and 12, lung fluid, tumor, and kidney specimens, respectively, from the same SPA-affected animal; lane 13, negative control; lane 14, pJS382 (JSRV *gag* clone); lane M, Boehringer marker VI.

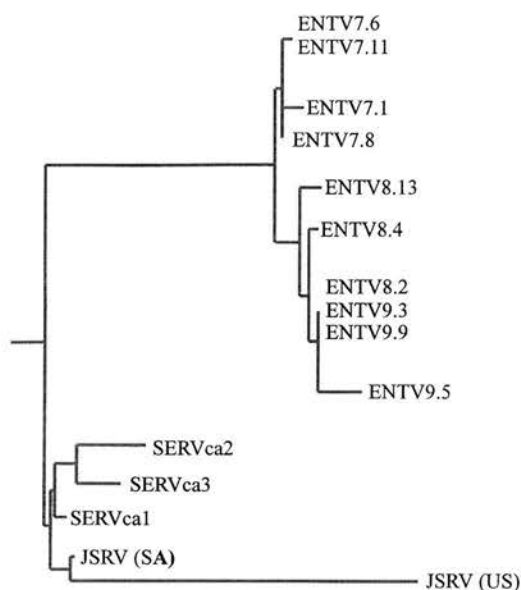


FIG. 3. Phylogenetic tree constructed from the sequences shown in Fig. 1.

from six ENT nasal fluid specimens (from four sheep and two goats) and six ENT tissue specimens (from four sheep and two goats) cut with both enzymes, demonstrating the presence of exogenous ENT. Amplimers from isopycnic gradient-purified fractions were expected to cut completely, as they should contain no endogenous sequences. However, restriction enzyme digestion was incomplete. The efficiency of the restriction enzymes was checked by digestion of PCR product amplified from plasmid pENTV7.6 (sequence shown in Fig. 1) and was found to be less than expected. Nevertheless, the results show that *AatII*- and *PstI*-sensitive product was detectable in all tumor and fluid samples from animals with ENT.

DNAs from tumor and kidney tissues of two sheep and two goats affected by ENT were used as templates for *gag* PCR followed by *PstI* or *AatII* digestion to determine whether these restriction sites were present in any of the related endogenous sequences. No *AatII*- or *PstI*-sensitive product was detected (results not shown).

Relationship between ENT, JSRV, and SERVs. Comparison of the 10 ENT sequences with the published sequences of JSRV and SERVs showed that the ENT sequences were 95 to 100% similar to each other, 83 to 92% similar to JSRV sequences, and 83 to 92% similar to SERV sequences. Figure 3 shows a phylogenetic tree constructed from the *gag* sequences. The long branch linking the ENT sequence cluster to the rest (SERV and JSRV) was significantly greater than 0 (length, 0.09676 nucleotide substitutions per position, i.e., about 10% sequence divergence or 22 nucleotide substitutions in 229 bp). The ordering of the *gag* fragments within the ENT cluster and within the SERV-JSRV cluster was not significant. The optimum Ts/Tv value was 7.0, suggesting that transitions are much more common than transversions (e.g., A→G is 14 times more likely than A→T). The relative rates at the three codon positions were 1:1:10, confirming that the first two codon positions are constrained relative to the third position (which is likely to be evolving at close to the neutral rate) and suggesting that the part of the CA protein encoded by this region of *gag* does not evolve rapidly.

DISCUSSION

We have previously reported that the presence of a *ScaI* restriction site within *gag* is a molecular marker for JSRV (15). Here, we report that the restriction sites *AatII* and *PstI* within *gag* act as unique molecular markers for ENT. *gag* RT-PCR analysis of samples from clinical ENT cases and from unaffected controls demonstrated that the virus transcripts distinguished by the *AatII* and *PstI* restriction sites were present exclusively and consistently in tumor tissues and exudates from ENT cases. ENT therefore appears to be a distinct virus associated with ENT. By analogy with JSRV, ENT is likely to be an exogenous virus as opposed to a reactivated endogenous virus. Our findings that no endogenously derived sequences containing *AatII* or *PstI* were detected at either the RNA or the DNA level support this notion but because of the limitations of sensitivity of the present technique are not conclusive. These limitations are highlighted by the fact that we were unable to demonstrate the presence of ENT provirus in tumor tissue DNA. This observation agrees with our findings for JSRV, for which provirus could not be detected in SPA tumor DNA by using the *gag* PCR-*ScaI* technique because of the high background of endogenous sequences (15). This problem was overcome by the development of primers specific for exogenous JSRV which enabled detection of proviral DNA in tumor tissue. These JSRV primers do not amplify ENT, and further sequence data for ENT are necessary in order to design specific primers and develop a more sensitive PCR for detection of ENT.

The fact that the *AatII* and *PstI* sites were conserved in ENT whereas the *ScaI* site is conserved in JSRV suggests that ENT and JSRV are distinct viruses. This notion is supported by the phylogenetic tree, which shows that the JSRV and SERV sequences are more similar to each other than they are to the ENT sequences. However, such short regions of sequence should not be overinterpreted. It is not possible to extrapolate the conclusion from this small region of sequence to predict differences in other regions of the viral genomes, as it is likely that the *gag* gene may be the region of greatest homology between the two viruses, especially since analysis of the sequences suggests that the *gag* region is not evolving rapidly. In addition, the CA proteins of JSRV and ENT, encoded by *gag*, are antigenically related (8). However, antigenic relatedness of other viral components has not yet been investigated. PCRs which amplify other regions of JSRV and of SERVs (long terminal repeat, *pol*, and *env*) did not amplify ENT (data not shown). Caution should be exercised in interpretation of negative PCR results since minor differences between two genomes can prevent efficient primer binding; nevertheless, this result suggests not only that other genes of ENT are less similar to those of JSRV and SERVs but also that ENT is less similar to SERVs than is JSRV. This agrees with the phylogenetic tree.

In sheep and goats, ENTs are clinically and pathologically identical (4, 5), but, until now, there have been no data to confirm that the same virus affects the two species. Our studies show that the *AatII* and *PstI* sites are present in the *gag* region of the viruses from ENT samples of both sheep and goats, suggesting that the viruses are highly related. Similar intranasal tumors in other animals including cattle, buffaloes, and pigs have also been reported (16), and the RT-PCR-restriction enzyme technique could be used to determine whether these tumors are also associated with ENT.

In conclusion, this is the first report of nucleic acid sequence from ENT, and it confirms, at the nucleic acid level, that this retrovirus is related to JSRV yet apparently distinct from it.

ENTV can be specifically identified by RT-PCR followed by *AatII* or *PstI* digestion, and, by this technique, ENTV was demonstrated exclusively and consistently in nasal tumor tissues and exudates from ENT-affected sheep and goats. The facts that SPA and ENT are both adenocarcinomas of secretory epithelial cells but target different areas of the respiratory tract and that JSRV and ENTV are also similar but distinct add further weight to the hypothesis that these retroviruses are the etiological agents of the tumors.

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